THE RETENTION OF NUTRIENTS IN CHEESE MAKING

IV. THIAMINE IN CHEDDAR CHEESE MADE FROM RAW AND PASTEURIZED MILK

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ONE FIGURE

(Received for publication April 1, 1946)

Studies of the fate of calcium, phosphorus and riboflavin in the manufacture of cheese have been reported recently (Irvine et al., '45a, b, c). The present report deals with the recovery of thiamine in Cheddar cheese made from raw and pasteurized milk, and its retention during ripening.

There have been relatively few values for the thiamine content of cheese reported in the literature, and the numbers of samples concerned have been very small. Booher and Hartzler ('39) reported a value of 42 μg per 100 gm in one sample of American Cheddar cheese assayed biologically. In studies on various types of cheese by a thiochrome procedure, Pyke ('39) found 12 μg of thiamine in 100 gm of Cheddar. Lane et al. ('42) and Cheldelin and Williams ('42) reported on American cheese. The former group, using a thiochrome method, found 42 μg in 100 gm of the edible portion and the latter workers found the thiamine content of one sample, as determined by the yeast-growth method, to be 26 μg per 100 gm of fresh cheese. Sullivan et al. ('43) did not include thiamine in their extensive study of water-soluble vitamins in various types of cheese.

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Until recently there was no published information on the fate of thiamine during the manufacture and ripening of cheese. While the present study was in progress, Dearden et al. ('45) reported on extensive studies of several vitamins, including thiamine, in the production of Cheddar, Cheshire and Stilton cheese from unpasteurized milk. Approximately 15% of the thiamine present in the milk was retained in the cheese, and no significant losses occurred during ripening for periods up to 42 weeks. The ranges of thiamine content in ripe Cheddar, Cheshire and Stilton were, respectively, 53 to 57, 43 to 46 and 70 to 80 μg per 100 gm.

Numerous observations on the effect of pasteurization on the thiamine content of milk have been reported, and there is considerable variation in the destruction of thiamine found in different studies. Henry and Kon ('38) and Houston et al. ('40) reported that commercial sterilization causes losses of, respectively, 30% up to 50%, and the latter group found 10% destruction of thiamine in commercial pasteurization. Elvehjem ('41) reported a loss of 25%. More recently, the holder process of pasteurization was found to destroy 9% (Holmes et al., '43) and the high-temperature-short-time pasteurization to destroy 3% of the thiamine (Holmes et al., '45). Kendall ('42) observed 10 to 20% loss in pasteurization by unspecified methods. Because of these variable results and also because of the current interest in the question of pasteurization of milk for cheese manufacture, it was thought advisable to include this aspect in the present studies.

EXPERIMENTAL

The experimental lots of cheese were manufactured on four occasions at intervals of approximately 2 months. On each occasion, a bulk lot of 1600 to 1700 pounds of fresh milk from the College herd of Holsteins, Ayrshires and Jerseys, was thoroughly mixed and divided into three approximately equal weighed portions. The first portion was used in the raw state, the second was pasteurized by the holder method at 143°F. for 30 minutes, and the third portion was pasteur-

ized by the "High-Short" method at 161°F. for 16 seconds. Details of the pasteurization methods and the methods of cheese manufacture have been presented previously (Irvine et al., '45a, b). Samples of milk plus starter were taken from each of the vats before rennetting, and samples of the first whey were also taken. The weight of cheese produced and the weight of the first whey were recorded. The press whey was not considered.

The cheese from each vat was divided into two lots, one of which was ripened at 40°F. and the other at 58°F. Samples were taken for analysis at 1 day, 14 days, and 1, 3, 6, 9 and 12 months. At 1 and 14 days, only one sample was taken for each vat, but at the other periods one sample was taken from each of the 40° and 50° ripening lots. The method of sampling has been outlined previously (Irvine et al., '45a).

METHODS OF ANALYSIS

The milk, whey and cheese samples were analyzed for total solids and thiamine. For the former the samples were prepared by thorough grinding in a kitchen food grinder. Moisture loss from weighed portions in aluminum moisture dishes was determined after 24 hours at 100°C. Since the thiamine analyses were carried out with some slight variations in technical details from standard methods, our technic is described in detail.

Method for thiamine

The samples of cheese for thiamine determination were finely grated through a bronze screen of 20-mesh. This resulted in a rather feathery material for extraction. After thorough mixing, 20 gm of this material was weighed into a 125-ml Erlenmeyer flask, and to this were added 75 ml N/10 H_2SO_4 and 6 ml of 2.5 M sodium acetate solution. The resultant pH of this mixture was approximately 4.8. Five ml portions of freshly prepared solutions of papain and of takadiastase were then added. These solutions contained 0.4 gm of enzyme in 5 ml, the takadiastase solution being pre-

pared simply by dissolving the required amount in water, the papain solution being prepared by the method suggested by Cheldelin et al. ('42).

The milk and whey samples were treated similarly except that 40 gm portions were used, and there were added 7.5 ml of N H_2SO_4 and sufficient water to bring the total volume of liquid up to approximately 75 ml. The buffer and enzyme additions were the same as for cheese samples.

After thorough mixing, the samples were incubated for 4 hours at 37°C. This incubation period was found to be just as efficacious in extracting free thiamine as longer periods up to 14 hours. There was a suggestion that a small progressive destruction of thiamine occurred in incubation for periods between 18 and 24 hours, and beyond 24 hours the destruction was very marked. Approximately 55% was lost in 36-hour incubation and 79% in 48-hour incubation. This behaviour in cheese samples is similar to that observed in milk by Sure and Ford ('43), who reported 46% destruction in pasteurized and unpasteurized cow's milk incubated without enzymes for 22 hours. These authors attributed the destruction partly to the effects of the milk ash.

Following incubation the samples were heated in flowing steam for 30 minutes to inactivate the enzymes. While still hot, the fat was separated from the samples. This was accomplished quite readily by the use of separatory funnels with stopcock openings sufficiently large to permit the passage of the flocculent cheese solids. The fat was washed twice with small portions of hot water, these washings being added to the fat-free extracts. When cool, the extract was adjusted to 100 ml with water, and, following the addition of 2 gm of filter-aid [1], filtered through a fluted no. 40 Whatman filter paper. The use of filter-aid in this way resulted in clear filtrates, and caused little or no loss of thiamine, whereas filtration through a layer of filter-aid on a Buchner funnel caused 25 to 30% loss.

[1] Johns-Manville Celite Analytical Filter-Aid.

Duplicate 30-ml aliquots of the clear filtrates were passed through Decalso columns. The adsorption and elution were conducted by the method of the U.S.P. ('43). Portions of a freshly-prepared standard thiamine solution in buffered H_2SO_4 solution were subjected to the adsorption procedure along with each group of samples. The thiochrome reaction was conducted on the standard and sample eluates by the U.S.P. method ('43).

The fluorescence was measured in a photoelectric fluorometer [2], and the thiamine contents of the samples were calculated by the use of the factor obtained from the concurrent standard portions.

RESULTS

The thiamine values for the liquid samples and the 1-day cheese were used to calculate the total amounts of the vitamin in each of the phases in the manufacturing process. These values together with the weights of the three phases are assembled in table 1. It will be noted, from the recovery figures for thiamine, that very little actual destruction of the vitamin occurred during the cheese-making process. In all vats of one series, T2, and in one vat (no. 8) of series T3, recoveries were somewhat low, but in view of the impossibly high recovery figures in series T4, these probably should be viewed as being largely the result of experimental errors which are magnified by the process of summation.

The last column in table 1 shows the amounts of the total thiamine content of the milk which were retained in the cheese. The mean retention for the twelve vats was 8.8%, considerably lower than that reported by Dearden et al. ('45) who found an average retention of approximately 15% of the thiamine. This retention, however, is still considerably greater than that which would be expected on the basis of aqueous dispersion of the thiamine, which, calculations reveal, would account for retention in the cheese of only 3.6 to 4.0% of the milk thiamine. This gives further support to the concept that

[2] Coleman Model 12.

some of the thiamine is present in milk solids in a bound form. A similar finding with respect to riboflavin was reported by Dearden et al. and by Irvine et al. ('45a). In the present results there is no indication that pasteurization influenced thiamine retention in the cheese.

TABLE 1

Thiamine fate in cheese-making.

BATCH NO. AND DATE OF MANUFACTURE	VAT. NO.	TREATMENT	MILK	WHEY	CHEESE	% RECOVERY		% MILK THIAMINE RETAINED IN CHEESE
						Weight	Thiamine	
T 1	1	Raw	600 [1]	537	59.5	99.4		
			89.7	80.2	9.7		100.0	10.8
May 30,	2	Holder	600	530	61.6	98.6		
1944			92.5	81.6	10.0		99.1	10.8
	3	H-S	515	457	53.1	99.1		
			79.3	70.4	7.69		98.5	9.7
T 2	4	Raw	540	483.5	52.8	99.3		
			88.2	70.2	6.21		86.7	7.0
Aug. 1,	5	Holder	540	484	53.0	99.5		
1944			84.4	70.2	6.97		91.5	8.3
	6	H-S	540	478	52.6	98.3		
			84.4	69.3	5.72		88.9	6.8
T 3	7	Raw	550	491	53.75	99.1		
			89.7	82.3	7.06		99.6	7.9
Sept. 27,	8	Holder	550	488	54.6	98.7		
1944			84.7	70.7	6.92		91.6	8.2
	9	H-S	550	491	54.3	99.2		
			92.2	82.3	5.91		95.7	6.4
T 4	10	Raw	550	490	54.75	99.1		
			72.7	68.0	7.03		103.0	9.7
Nov. 22,	11	Holder	550	486	53.75	96.3		
1944			64.0	61.7	6.82		107.0	10.7
	12	H-S	550	489	54.3	98.8		
			73.4	69.2	6.97		104.0	9.5

[1] In each case the upper figure is the weight in pounds and the lower figure gives the milligrams of thiamine contained in the indicated weight of milk, whey or cheese.

TABLE 2

The effect of ripening on the thiamine content of cheese.

BATCH NO. AND DATE OF MANUFACTURE	VAT NO.	TREATMENT	RIPENING TEMPERATURE °F	THIAMINE IN MILK (μG/100 GM)	THIAMINE IN CHEESE (μG/100 GM)[1]						
					1 day	14 days	1 month	3 months	6 months	9 months	12 months
T 1	1	Raw	40	33	36	34	26	26	25	24	21
			58		..	..	20	16	14	15	15
May 30, 1944	2	Holder	40	34	36	35	30	27	17	26	28
			58		..	..	28	19	21	20	18
	3	H-S	40	31	32	31	28	22	23	19	19
			58		..	..	27	15	17	14	13
T 2	4	Raw	40	36	26	28	26	25	20	17	9
			58		..	..	18	14	10	8	5
August 1, 1944	5	Holder	40	35	29	33	32	30	24	17	10
			58		..	..	28	22	21	22	7
	6	H-S	40	35	24	29	27	20	23	19	10
			58		..	..	22	25	13	12	7
T 3	7	Raw	40	36	29	34	30	30	20	9	12
			58		..	..	26	19	16	6	7
Sept. 27, 1944	8	Holder	40	34	28	41	33	34	30	12	14
			58		..	..	32	29	20	9	11
	9	H-S	40	37	24	32	27	30	20	9	11
			58		..	..	25	20	16	7	7
T 4	10	Raw	40	29	28	24	26	22	11	10	10
			58		..	..	25	16	5	11	4
Nov. 22, 1944	11	Holder	40	26	28	32	30	25	15	12	..
			58		..	..	25	19	11	12	10
	12	H-S	40	29	28	27	23	18	12	10	11
			58		..	..	22	16	7	7	8

[1] Content expressed in terms of cheese of 35% moisture content.

The thiamine values for the milk and for the various lots of cheese at different stages of ripening are presented in table 2. The values for the milk suggest that very little, if any, destruction of thiamine occurred in either pasteurization process.

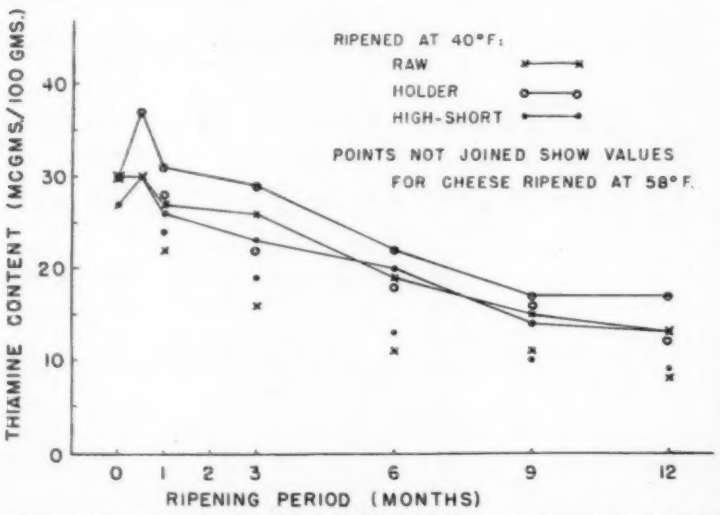

Fig. 1 Graph showing decrease in thiamine content of Cheddar cheese during ripening. The values shown are the averages for four batches and have been adjusted to the basis of 35% moisture content.

The thiamine content of the cheese samples showed a progressive decrease during ripening. This was true at both ripening temperatures, but invariably the thiamine loss was greater in the cheese ripened at 58°F. The average losses in 12 months ranged from 43 to 52% for 40° ripening [3] and from 60 to 73% for ripening at 58°F. These progressive losses are readily seen from the curves in figure 1 in which have

[3] A recent regulation of the Canadian Government forbids the sale of pressed cheese which has been ripened at a temperature below 45°F. This and similar regulations in force in many states of the United States are designed to eliminate cheese as a carrier of typhoid fever organisms. The present work, initiation of which antedated the Canadian regulations, should not be construed as an argument in favor of 40°F. ripening at the risk of typhoid contamination.

been plotted the averages of the four batches of cheese. It will be noted that the thiamine levels are highest throughout ripening in the cheese manufactured from milk pasteurized by the "Holder" process, the situation being the same for both ripening temperatures. This relationship is substantiated by the fact that graphs plotted for the individual batches, but not presented herewith, show this same behavior in all cases with but one possible exception. In these individual graphs, too, it is observed that the thiamine levels for the "raw" and "high-short" samples are, in general, very close together, appreciably lower than those of the "Holder" samples. These facts suggest that the thiamine decrease on ripening may be influenced by some catalytic factor which is inactivated at least partially by the "Holder" method of pasteurization but not by "High-Short" pasteurization. The authors can suggest no satisfactory explanation for the apparent increase at 14 days which is shown by the composite figure 1 and by the data for many of the individual batches.

SUMMARY

The fate of thiamine in the manufacture of Cheddar cheese and the effect of ripening periods up to 12 months upon this vitamin in the cheese produced have been studied. Cheese was manufactured at four 2-month intervals, covering the spring, summer and fall seasons. On each occasion, raw milk and milk pasteurized by two different methods were used for the cheese manufacture.

Milk pasteurized by either the "Holder" or the "High-Short" process showed little or no loss of thiamine.

The process of cheese manufacture caused no actual destruction of thiamine. The average retention in the cheese of this vitamin present in the milk was 8.8%. This is higher than can be accounted for on the basis of water-solubility, indicating that some of the thiamine is "bound" to some portion of the milk solids, presumably protein.

A progressive decrease in thiamine content of the cheese with increasing ripening period was observed. The losses in

12 months ranged from 43% to 73%. Ripening at 58°F. caused greater decreases than ripening at 40°F.

Cheese manufactured from milk pasteurized by the "Holder" process showed higher thiamine levels throughout ripening than the cheese from raw or "High-Short" pasteurized milk.

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REPRODUCTIVITY AND GROWTH OF ALBINO RATS ON A PROLONGED DAILY INTAKE OF CAFFEINE

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ONE FIGURE

(Received for publication April 29, 1946)

Studies made by different investigators on the effect of caffeine on reproductivity and growth have led to divergent conclusions. Stieve ('30, '31) described degenerative changes in the testicles of rabbits that had been given caffeine in a coffee brew by stomach tube for 3 to 4 weeks. These changes he believes accounted for the smaller number of offspring delivered by the females in his test group on coffee as compared with the controls given an equal amount of Kaffee Hag containing only a minute amount of caffeine. Vacca ('26) had previously reported a deleterious action of caffeine on the dog's testicle. Eichler and Mügge ('32), however, found that daily subcutaneous injections of caffeine did not result in a diminution of the reproductive capacity of albino rats over four generations. Their observations did not extend over the entire reproductive period of any one of the four generations. Hammill and Miller ('35) reported that litters of their test animals given caffeine containing beverages were of normal size. At variance with these results are the preliminary observations of Cheney ('44) indicating a reduced fecundity in rat colony studies extending over a period of 5 years.

As regards growth, some investigators (Hammill and Miller, '35; Smith and Hambourger, '36) report that prolonged

administration of caffeine retards it. Others (Stieve, '30; Eichler and Mügge, '32) found that the drug had no effect. In the experiments of Scott and Chen ('44) there was no depression in growth when the food mixture given the animals contained caffeine in a concentration as high as 0.1%.

The indecisiveness of these studies suggested the advisibility of the present investigation. Because of the extensive use of the caffeine-containing beverages, coffee and tea, the problem assumes practical interest from the nutritional point of view.

PROCEDURE

Twenty-five groups of albino rats were set apart at weaning for the experiment. Each group consisted of two males and two females from the same litter. One pair (male and female) served as test animals, the other pair as controls. All the animals were fed the same ration of commercial dog food [1] supplemented with green food and cod liver oil and were allowed to eat ad libitum. The drinking fluid to which there was free access at all times was tap water for the controls and a sweetened beverage containing caffeine for the test animals. This beverage constituted the sole fluid intake from the time of weaning to the conclusion of the experiment. It was chosen in preference to a solution of caffeine in water in order to insure as large an intake of caffeine as possible. Preliminary experimentation had revealed that the animals would drink a much larger quantity of this beverage than a solution containing the same amount of caffeine in plain water. The fluid was placed in an inverted graduated test tube provided with a drinking tip to which the animal had ready access. Various tests and observations had shown that there was no loss of fluid when the apparatus was left undisturbed and an extremely small loss when the animal was drinking. This was estimated to be considerably less than 5% of the contents of the container. The fluid intake of the adult control animals was approximately 30 ml per day

[1] Purina Dog Chow.

and that of the test animals 60 ml. This volume of the caffeine solution contained 12 mg caffeine which gave an average daily intake of approximately 40–50 mg per kilo body weight.

When the animals were 100 days old they were mated, a control male with a control female and a test male with a test female. Each pair was kept in a separate cage throughout the experiment. Records were kept on the date of birth of each litter, the number of offspring and the number of young that survived to weaning at the age of 21 days. The experiment was terminated for the individual pairs of animals when the females failed to become pregnant within 3 months after the birth of the last litter. The animals were then sacrificed and the testicles of the males removed and prepared for histological examination. The experiment was carried on continuously over 3½ years.

RESULTS

Reproductivity

The experimental data presented in table 1 show that there was considerable variation in the number of litters and number of offspring in each litter among both test and control animals. The averages were slightly higher in the test than in the control animals but the differences were not statistically significant. The critical ratio was only 1.3 times the difference.

The control animals gave birth to 105 litters containing a total of 618 offspring, or an average of 5.9 per litter; the test females delivered 135 litters containing 714 offspring, or an average of 5.3 per litter. Two of the control females produced no offspring whereas all of the females of the test group had at least one litter. There were four females in the control group and one in the test group that failed to raise a single animal to the weaning stage. 52.2% of the total number of offspring of the test animals and 53.2% of the controls survived to weaning. The average length of time between mating and delivery of the first litter was 31 days for the controls and 29 days for the test animals.

TABLE 1

Reproductivity of albino rats on a caffeine-containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

	TESTS			CONTROLS		
Group number	Number of litters	Number of offspring	Number weaned	Number of litters	Number of offspring	Number weaned
1	3	29	13	1	5	0
2	5	16	0	6	40	32
3	11	71	47	6	34	22
4	4	32	20	14	74	33
5	4	28	9	1	3	0
6	4	14	1	0	0	0
7	9	32	7	6	39	18
8	11	45	33	6	45	39
9	7	50	19	3	17	8
10	9	58	37	13	76	61
11	1	8	4	2	10	6
12	4	27	22	2	8	3
13	4	23	16	3	24	9
14	1	5	5	7	44	3
15	10	42	27	4	18	16
16	4	18	4	2	9	0
17	7	32	12	6	34	24
18	3	13	9	1	8	0
19	1	8	5	2	19	5
20	6	27	17	3	18	11
21	9	30	11	3	19	5
22	5	27	10	1	9	2
23	7	52	27	7	29	12
24	3	15	10	0	0	0
25	3	12	8	6	36	20
Total	135	714	373	105	618	329
Average per group	5.4	29	15	4.2	25	13

Testicular histology

A histological examination [2] was made on the testicles of twenty-three of the test males and fifteen controls. The find-

[2] All histological studies were done by Dr. Walter Sheldon, pathologist at Grady Hospital, Atlanta, Ga.

ings on 57% of the tests and 53% of the controls were essentially negative. In the remainder there occurred certain histological changes which, however, were of the same type in both the tests and controls. None of the lesions resembled those described by Stieve in rabbit's testicles. In some of the testicles small patchy areas were observed in which the seminiferous tubules had become atrophic. These tubules were lined by a thin, irregular layer of atrophic epithelium suggesting fatty degeneration. There was no trace of spermatogenesis left. In these areas there was some increase in the interstitial cells but no appreciable thickening of the tubular basement membrane. The remaining portions of the testes were normal with normal spermatogenesis. In other testicles there was a slight general reduction in spermatogenesis but no areas of atrophy. Several showed extensive areas of tubular atrophy and fibrosis with reduced spermatogenesis in the remaining tubules. These changes may be accounted for by spontaneous physiological involution.

No relationship could be detected between the histological changes in the testicles and the number of offspring. For example, in animal no. 17 of the test group, the testicles showed fairly extensive lesions and in animal no. 20 the lesions were very slight. However, the former sired seven litters containing a total of thirty-two young, while the latter sired only six litters containing twenty-seven young. In both these instances the number of offspring was larger than in many cases in which no histological changes were seen in the testicles.

Growth

Weekly weighings were made on ten test and ten control males for 26 weeks and on an equal number of females for 11 weeks. The weighings were discontinued on the females at this time because they were then mated and became pregnant shortly thereafter. From the weight curves presented in figure 1 it is apparent that the daily ingestion of caffeine had no retarding effect on growth.

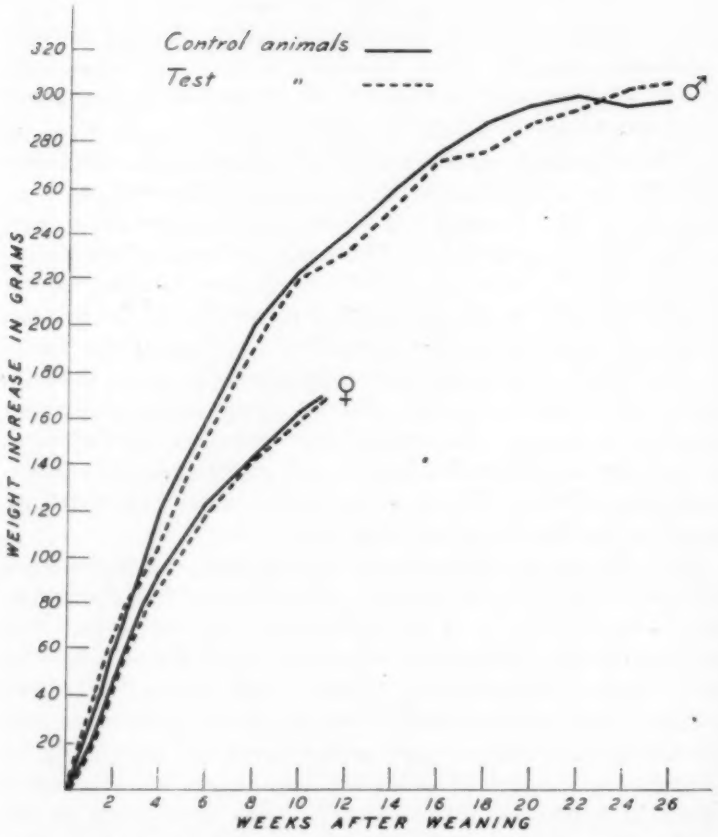

Fig. 1 Growth curves of albino rats on a caffeine containing beverage as their sole fluid intake, as compared with their litter mate controls on water.

DISCUSSION

In the experiments of Eichler and Mügge ('32) it was found that the prolonged administration of caffeine to albino rats produced no diminution in reproductive capacity over four generations. Experimentation on each generation was terminated after the birth of the first litters. From our observa-

tions on both the number of offspring and the testicular tissue of the male parents, it is apparent that throughout the reproductive period of the animals' life there was no evidence of sterility as a result of the daily ingestion of relatively large amounts of caffeine.

The discrepancy between our observations and those of Stieve ('30, '31) on the testicle of the rabbit and those of Vacca ('26) on the dog's testicle might conceivably be due to a species difference in response to caffeine. This explanation, however, can not be accepted without further experimentation. The experiments of Stieve, as well as those of Vacca, appear to have been poorly controlled. The only information available with regard to Stieve's animals is that they were pure blooded Russian rabbits, 7 to 16 months old with an average weight of 1865 gm for the females and 1887 gm for the males. In studies of this kind it is of paramount importance that environmental and nutritional conditions be the same for both the test and control animals before and during the experiment, for it has been shown that such factors as light (Fiske, '39, '41; Pomerat, '42) and vitamin deficiencies and inanition (Mason, '30, '33; Mason and Wolfe, '30; Truscott, '44) affect the pituitary which, as is well known, exercises through its gonadotropic hormone a direct action on the testicles. In Vacca's experiments ('26) not only were the four dogs used as test animals selected at random but also the amount of caffeine administered was obviously toxic. All the test animals became emaciated and had general tremors and convulsions.

The difference in our results as compared with those obtained by Hammill and Miller ('35) with respect to growth may be due to the fact that in their experiments the various groups of animals were not fed the same diet. The authors state that all the diets, the composition of which is not given, were satisfactory for experimental purposes, but it is possible that they might not have been equally effective in promoting growth.

This objection does not apply to the observations of Smith and Hambourger ('36). However, it should be noted that their animals were given 100 mg caffeine per kilo by stomach tube whereas ours ingested ad libitum the smaller amount of 40 to 50 mg per day. It is hoped that further experimentation now under way in our laboratory may show whether the differences between our results and theirs were due to the different amount of caffeine the animals received or the different experimental procedure.

SUMMARY

Albino rats were given a sweetened caffeine beverage as their sole source of fluid throughout their reproductive period.

The average daily consumption of caffeine by each animal amounted to 40–50 mg per kilo body weight.

The reproductive capacity as judged by the number of litters and number of offspring was not impaired by the daily ingestion of caffeine.

There were no pathological changes in the testicles of the test animals except for the natural histological degenerative changes which were observed in about equal frequency in the control animals.

The rate of growth of the animals on the caffeine beverage was the same as that of the controls.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

VIII. THE UTILIZATION OF THIAMINE DURING LACTATION [1]

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EIGHT FIGURES

(Received for publication May 20, 1946)

The metabolism of thiamine during the reproductive cycle, especially during lactation, is largely a matter of conjecture. In recommending dietary allowances for lactating women, the Food and Nutrition Board of the National Research Council ('45), after reviewing the published data dealing with thiamine requirement, suggested that 2.0 mg of thiamine be allowed for a diet containing 3000 cal. This thiamine to calorie ratio of 0.67 is somewhat higher than the 0.5 mg thiamine per 1000 cal.[3] recommended for normal adults. It was increased because additional thiamine may be needed for the synthesis of milk, to give added protection during the child-bearing period. Some believe that the allowance has been placed too high, but as the Food and Nutrition Board pointed out, the recommendations do not constitute requirements, but were selected to provide margins of safety. Variations in requirements are known to be large owing to differences in size and

[1] The investigation represented in part by this paper was partially supported by a grant from The Nutrition Foundation, Inc., and was made possible by the cooperation of J. P, Pratt, M.D., Chief of the Department of Obstetrics, B. M. Hamil, M.D., Departmet of Pediatrics, Elizabeth Moran, R.N., Director of Nurses, and Annie Lou Wertz, B.S., dietitian, all of the Henry Ford Hospital, Detroit.

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[3] Kilogram calories.

absorption as well as efficiency of utilization, and the margins of safety are for those whose requirements are above the average.

There is evidence that the non-fat calories of a diet may be a better basis for estimating thiamine requirement than the total calories. Reinhold, Nicholson and Elsom ('44) found that increased amounts of thiamine were utilized when the proportion of carbohydrates in the diet was high. The so-called "sparing action" of fat appears to be related to a coincident decrease in the carbohydrate content of a diet. The decreased thiamine excretion on a high carbohydrate diet is interpreted as indicating an increased need for thiamine in the metabolism of the carbohydrate; therefore, the proportion of carbohydrate in the diet may be an important factor in determining the daily need for thiamine. Perhaps weight or surface area should also be considered. Elsom, Reinhold, Nicholson and Chornock ('42) in studies of the B vitamins fed six human subjects thiamine in amounts consistent with the theoretical requirement based on Cowgill's ('34) formula and found that three subjects developed deficiency symptoms. These were the three smallest people in the group and this prompted Elsom to conclude that smaller people eating less food and therefore receiving fewer calories may have higher requirements per unit of body weight than larger people. Recent work of Ayre and Bauld ('46) has emphasized the importance of adequate thiamine intake: "The finding of abnormal estrogenic activity coupled with thiamine deficiency in cases of menorrhagia and uterine cancer suggests a possible etiological correlation between the dietary deficiency, the abnormal estrogen level, and the pathological lesion."

EXPERIMENTAL PROCEDURE

By chemical analysis, the thiamine intakes of nursing mothers at various stages of lactation have been determined, as well as their daily thiamine excretions in urine and secretions in breast milk during the same periods. No attempt was made to collect feces since, although it is known that

bacteria synthesize the B vitamins in the intestinal tract, it is not known whether this material is available to the human organism. Most of the experimental evidence indicates that (a) fecal thiamine is unrelated to the intake (Leong, '37), except in cases of very large intakes, (b) it is chiefly present in the bacterial cells and unavailable to the animal organism (Abdel-Salaam and Leong, '38; Emerson and Obermeyer, '45), and therefore, it is probably insignificant in studies on normal diets.

The subjects of the investigation were multiparas with medical records of good or excellent health and having successfully nursed their other children. Of the fourteen women participating in the study seven were studied during two consecutive 5-day periods immediately following delivery. Five were observed for 1 day, 7 to 9 days postpartum. Ten of the fourteen mothers were studied during eighteen 5-day periods at various intervals during the production of mature milk. All of the diets were comparable, qualitatively, but the amounts of all foods except milk were increased or decreased so that the appetites of the women were satisfied. Duplicates of each meal were added to composites which were analyzed for each 5-day period. Complete 24-hour collections of milk and urine were obtained. Details of selection of subjects, and the methods of manually expressing the milk and of collecting the food and milk are given in preceding publications (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Davies, '45). In conjunction with many periods during which mature milk was collected, fasting samples of urine were obtained the first morning of the 5-day period and the morning following the period. Each collection represented excretion for 1 hour, 12 to 13 hours following the last meal. Subsequent papers will present the data for other vitamins and minerals.

Sample preparation and analysis

Food. Approximately 400 gm of the ground, 5-day food composite was homogenized in a Waring Blendor and the

volume made to 500 ml. Of this, 20 ml were used for each of duplicate determinations. Each sample was diluted to 75 ml with water and enough 1 *N* sulfuric acid to make the mixture 0.1 *N* and autoclaved for 10 minutes at 15 pounds pressure. When cool, the solution was adjusted to pH 4.5 with 2.5 *M* sodium acetate, then incubated under toluene with clarase overnight (about 16 hours) at 37°C. Finally, the sample was diluted to 100 ml, filtered, and 40 ml of the filtrate used for the thiamine determination. Riboflavin was determined in the remaining filtrate (Roderuck, Coryell, Williams and Macy, '45).

Milk. An aliquot of milk from each expression during a 24-hour period was poured immediately into a jar which was kept tightly covered in a refrigerator. The composite for the day preceding was taken to the laboratory each morning and held under refrigeration pending analysis by the method of Hennessy ('42) slightly modified (Roderuck, Williams and Macy, '45). The majority of the assays were made within 24 hours.

Urine. Each voiding of urine was added to an amber glass bottle containing acetic acid and toluene. The 24-hour composite was 2% acetic acid. Each day the composite was taken to the laboratory, the volume recorded and aliquots removed for analysis by the thiochrome procedure (Roderuck, Coryell, Williams and Macy, '45). Filtration was the only pretreatment required with urine, since all the thiamine was in the free state, so measured amounts of filtered urine were added directly to Decalso columns. Usually 10 ml per liter of urine excreted was used for each determination. The sulfite treatment described by Mason and Williams ('42) was satisfactory in obtaining blank determinations when 50 mg of sodium sulfite was used for each sample, instead of the 25 mg which they recommended.

RESULTS AND DISCUSSION

Table 1 gives for all the women studied during the first 10 days postpartum the daily volumes of milk and urine,

the average daily thiamine intakes, the daily secretions of free and total thiamine in milk, and the daily excretion of thiamine in the urine. Averages of all values obtained for each of the 10 days are given in table 2. While only insignificant amounts of thiamine were secreted in the colostrum during the first 2 days postpartum, the rapid increases in immature milk volume, with greater concentration (Roderuck, Williams and Macy, '45), resulted in an average daily secretion of 95 μg on the tenth day. Although larger amounts of free thiamine also were secreted, the major portion of the increases in the amounts of total thiamine in milk during the puerperium consisted of bound thiamine. A preceding paper (Roderuck, Williams and Macy, '45) pointed out that the concentration of total thiamine in colostrum during the first 4 days of lactation varied from 0.9 to 2.4 μg, increasing to an average of 8.1 μg per 100 ml on the tenth day. The average concentration of free thiamine was not more than 0.5 μg the first 6 days of lactation and the highest value was 1.0 μg on the ninth day postpartum.

Within the range of thiamine intakes in this study there was no relationship between thiamine intake and secretion in milk. The scatter diagrams in figures 1 and 3 demonstrate the increases in milk volume and secretion of thiamine in milk during the first 10 days postpartum. The data in tables 1 and 2 and the values plotted in figures 1 to 8 inclusive show no relationship among thiamine intakes, urine volumes and levels of thiamine excretion in urine. The values for the different women (table 1) show parallel trends in increasing milk volume and thiamine secretion in milk during the first 10 days postpartum.

The average values obtained for each 5-day period during the puerperium and during mature milk production are given in table 3. The daily milk and urine volumes and the percentages of the average thiamine intake secreted in milk and excreted in urine are plotted in the figures 1 to 8. Although evidence obtained with rats has shown that additional thiamine is required to support satisfactory milk production

TABLE 1

Thiamine intakes, excretion in urine and secretion in immature milk during first 10 days postpartum.[1]

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE			
					Milk		
		Milk	Urine	Intake	Total	Free	Urine
	days	*ml*	*ml*	*mg*	*μg*	*μg*	*μg*
L.F.	1	71	2590	1.10	1	0	315
	2	200	1275	1.10	2	2	215
	3	733	2420	1.10	9	2	285
	4	1122	2075	1.10	19	6	210
	5	1441	1674	1.10	25	9	225
	6	1501	1718	1.12	35	10	192
	7	1596	1340	1.12	49	11	150
	8	1638	1810	1.12	64	13	149
	9	1676	1374	1.12	105	21	138
	10	1872	2220	1.12	129	20	172
V.G.	1	82	2067	0.82	2	..	104
	2	177	1689	0.82	3	1	107
	3	849	1775	0.82	16	3	113
	4	1413	1323	0.82	25	1	108
	5	1471	1303	0.82	27	2	103
	6	1782	1148	0.73	47	4	93
	7	1630	1230	0.73	59	3	109
	8	1895	1237	0.73	97	7	95
	9	1828	1104	0.73	106	12	118
	10	1770	1075	0.73	120	13	95
V.K.	1	9	3086	0.92	..	..	146
	2	90	3651	0.92	1	..	187
	3	484	2596	0.92	6	..	126
	4	547	2427	0.92	10	..	141
	5	560	2331	0.92	15	..	134
	6	663	2249	0.98	23	3	164
	7	781	3380	0.98	40	4	183
	8	775	3840	0.98	51	6	156
	9	794	3115	0.98	59	8	151
	10	797	3328	0.98	65	7	148
V.L.	1	30	1757	1.25	..	..	196
	2	56	2686	1.25	..	..	188
	3	353	2759	1.25	6	1	171
	4	794	1823	1.25	14	2	170
	5	844	1847	1.25	18	3	137
	6	955	2710	1.34	33	4	196
	7	1047	2841	1.34	47	3	255
	8	1098	2066	1.34	59	12	209
	9	1118	2151	1.34	75	7	174
	10	1200	1669	1.34	80	10	162

TABLE 1 — (*continued*)

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	*ml*	*ml*	*mg*	*µg*	*µg*	*µg*
J.M.	1	35	2182	1.15	...	...	60
	2	385	1386	1.15	6	1	108
	3	870	1519	1.15	14	2	94
	4	1011	1201	1.15	24	3	94
	5	1121	1283	1.15	38	4	90
	6	1125	1172	1.25	51	6	101
	7	1287	1161	1.25	76	7	169
	8	1136	1520	1.25	89	10	141
	9	1258	...	1.25	112	11	...
	10	1336	1553	1.25	136	12	148
C.O.	1	16	1864	1.00	...	...	67
	2	100	939	1.00	1	...	62
	3	335	1511	1.00	4	0	87
	4	595	....	...	7	3	...
	5	725	....		13	4	...
	6	821	...	1.05	11	4	...
	7	798	1470	1.05	24	6	121
	8	950	1127	1.05	39	5	87
	9	931	1191	1.05	42	9	104
	10	660	....	1.05	43	11	...
V.S.	1	6	1869	1.31	...	...	513
	2	92	1874	1.31	2	...	444
	3	420	2887	1.31	9	1	390
	4	600	2063	1.31	14	2	288
	5	697	2065	1.31	22	2	261
	6	756	1502	1.32	37	5	249
	7	818	2524	1.32	51	6	352
	8	837	2237	1.32	64	10	296
	9	932	1984	1.32	82	10	284
	10	924	2287	1.32	92	11	264
M.B.	9	1189	1035	1.28	125	23	47
E.L.	8	688	677	1.06	22	6	72
M.S.	7	880	1355	0.88	43	10	81
G.S.	7	1017	1981	1.04	40	5	146
F.W.	7	953	1032	1.23	44	7	74

[1] The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F., 23.5 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L., 5 hours; J.M. 16 hours; and V.S. 7 hours.

(Macy, Outhouse, Long and Graham, '27), in the present investigation diets which supplied amounts of thiamine much lower than the Recommended Dietary Allowances of the Food and Nutrition Board ('45) provided adequate amounts for the mothers' bodies and for milk production without depressing the rates or amounts of urine excretion. It should be

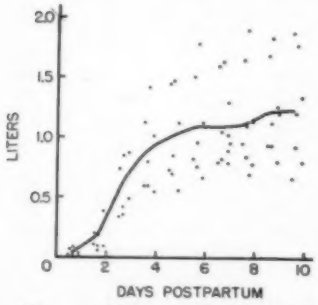

Fig. 1 Volume of immature milk secreted per 24 hours.

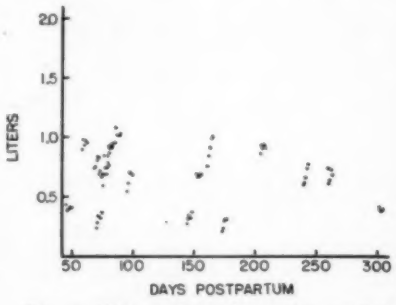

Fig. 2 Volume of mature milk secreted per 24 hours.

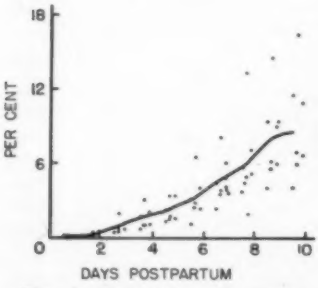

Fig. 3 Percentage of average thiamine intake during 5 days secreted into 24-hour collections of immature milk.

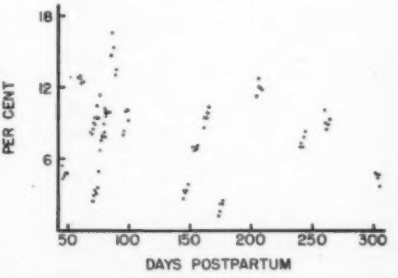

Fig. 4 Percentage of average thiamine intake during 5 days secreted into 24-hour collections of mature milk.

pointed out that the volume of milk during the last few days of the lying-in period is largest, but the concentration at that time is small, so that the total daily output is also small. The concentration of thiamine, as pointed out in an earlier paper (Roderuck, Williams and Macy, '45), remained rela-

tively constant from the fifth week of lactation until milk flow diminished. The average daily secretion in mature milk seems to have been related to milk volume rather than to thiamine intake. Even during the second to third month of

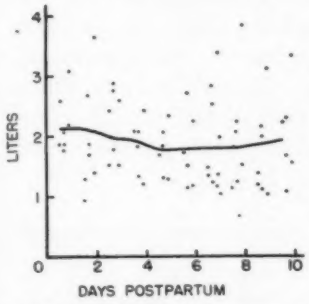

Fig. 5 Volume of urine per 24 hours during period of secretion of immature milk.

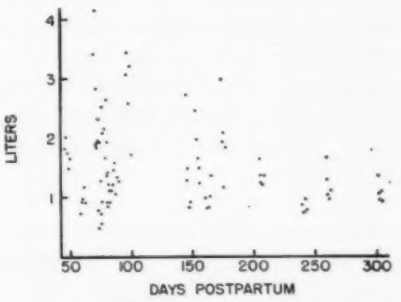

Fig. 6 Volume of urine per 24 hours during period of secretion of mature milk.

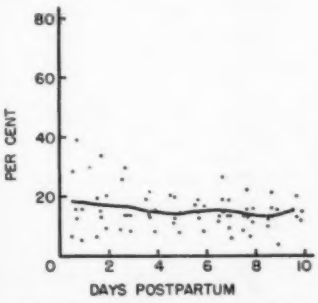

Fig. 7 Percentage of average thiamine intake during 5 days excreted in 24-hour collections of urine during period of secretion of immature milk.

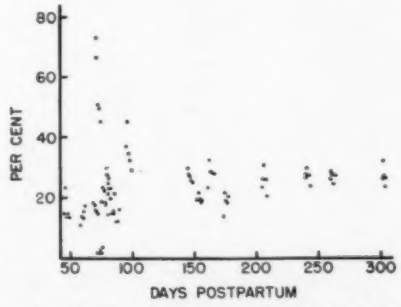

Fig. 8 Percentage of average thiamine intake during 5 days excreted in 24-hour collections of urine during period of secretion of mature milk.

lactation, which is the period of highest secretion of thiamine in the milk, all of the averages for thiamine output in milk were less than 15% of the intake (fig. 4). Perhaps, then, in lactation the thiamine requirement is increased only by this amount.

That the largest percentages of thiamine unaccounted for in milk and urine occurred in the 5-day period following parturition suggests that muscle metabolism during labor may have drawn extensively on the mothers' thiamine stores, producing a need for replenishment of their reserves. The thiamine concentration in blood has been shown to be lower after birth than at the end of pregnancy (Gaehtgens and Sauerbrey, '43; Neuweiler, '42). The average daily intake of thiamine was somewhat lower for this period than for any other period and therefore may have been utilized more efficiently.

TABLE 2

Thiamine in 24-hour collections of immature milk and urine during the first 10 days postpartum.

INTERVAL POSTPARTUM	MILK			URINE		
	Volume	Thiamine		Volume	Thiamine	
days	*ml*	*μg/day*	*μg/100 ml*	*ml*	*μg/day*	*μg/100 ml*
1	36	1	1.5	2202	200	9.6
2	157	3	1.4	1928	187	10.5
3	578	9	1.6	2210	181	7.8
4	869	16	1.8	1819	168	9.2
5	980	23	2.4	1750	158	9.0
6	1086	34	3.2	1750	166	9.8
7	1081	47	4.5	1831	164	9.2
8	1127	61	5.5	1814	151	8.9
9	1216	88	7.4	1708	145	8.7
10	1223	95	7.9	2022	165	8.6

During the sixth to tenth days, 13–15% of the thiamine appeared in the urine, approximately the same percentage as during the first 5 days postpartum, but the milk thiamine increased from 3.0 to 8.5% of the intake. In the periods of mature milk secretion the percentages of thiamine in milk increased only slightly, whereas the percentages of thiamine in the urine increased noticeably which may indicate greater saturation of the mothers' tissues as lactation progressed.

The average percentage of thiamine intake accounted for during the mature milk periods was 31%; of this, 23%, an average of 305 μg per day, appeared in the urine. According

TABLE 3

Average daily thiamine intakes, excretion in urine and secretion in milk during thirty-two 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		THIAMINE						
		Milk	Urine	Intake	Milk				Urine	
					Total		Free			
	days	*ml*	*ml*	*mg*	*μg*	*% intake*	*μg*	*% intake*	*μg*	*% intake*
V.G.	1–5	798	1631	0.82	14	1.7	2[1]	0.2	107	13
	6–10	1781	1159	0.73	86	11.8	8	1.1	102	14
	78–82	848	1281	1.36	126	9.2	33	2.4	318	23
	161–165	901	993	1.18	112	9.5	90	7.6	331	28
	239–243	681	810	1.18	89	7.5	81	6.8	320	27
	302–306	394	1080	1.38	62	4.5	58	4.2	375	27
V.K.	1–5	338	2818	0.92	6[1]	0.6	..	..	147	16
	6–10	762	3182	0.98	48	4.9	6	0.6	160	16
	95–99	647	2804	1.07	96	9.0	23[1]	2.1	382	36
	144–148	325	1447	1.20	39	3.2	11	0.9	326	27
V.L.	1–5	415	2174	1.25	..	..	..	..	173	14
	6–10	1084	2287	1.34	59	4.4	7	0.5	199	15
	68–72	789	2849	1.50	127	8.5	25[1]	1.7	243	16
	152–156	680	1756	1.61	111	6.9	40	2.5	315	20
J.M.	1–5	684	1514	1.15	16[1]	1.4	3[1]	0.3	89	8
	6–10	1228	1352	1.25	93	7.4	9	0.7	140[1]	11
	75–79	708	2265	1.38	97	7.0	24[1]	1.7	286	21
	173–177	268	2003	1.59	30	1.9	10	0.6	297	19
B.S.	85–89	1020	1347	1.11	162	14.6	40	3.6	169	15
	204–208	913	1356	1.32	158	12.0	75	5.7	333	25
	259–263	676	1219	1.26	115	9.2	63	5.0	336	27
V.S.	1–5	363	2152	1.31	9[1]	0.7	2[1]	0.2	379	29
	6–10	853	2107	1.32	65	4.9	8	0.6	289	22
	70–74	304	1852	1.40	43	3.1	24	1.7	798	57
L.F.	1–5	713	2007	1.10	11	1.0	4	0.4	250	23
	6–10	1657	1692	1.12	76	6.8	15	1.3	160	14
C.O.	1–5	354	1438	1.00	5[1]	0.5	2[1]	0.2	72[1]	7
	6–10	832	1263	1.05	32	3.0	7	0.7	104[1]	10
M.B.	72–76	718	691	1.06	106	10.0	41	3.9	27	2
D.M.	45–49	401	1741	1.36	65	4.8	35[1]	2.6	217	16
M.S.	58–62	947	939	1.17	147	12.6	39	3.3	165	14
G.S.	80–84	899	1078	1.41	132	9.4	40	2.8	256	18

[1] Less than five 24-hour collections included in average. For total thiamine in milk values were not obtained for first day but values calculated from concentration in milk of second day are included in average (table 1).

to values in the literature for excretion by normal adults, the nutritional status of the mothers studied was satisfactory with respect to thiamine. The average values for urine in the two periods during the puerperium, 174 and 165 μg per day, respectively, also exceed the figures which are considered normal. Toverud ('39) found an average excretion of only 70 μg per 24 hours from lactating women who were receiving a diet calculated to provide 1.5 to 2.0 mg per day. Melnick, Field and Robinson ('39) found that normal women excreted about 90 μg per day, or 14% of their intake. This figure is lower than their average for normal males, who excreted 198 μg per day, 20% of their intake, and is considerably lower than the values found in this study. Jolliffe, Goodhart, Gennis and Cline ('39) found that 7 to 24% of the thiamine intake appeared in the urine. Normal individuals, according to Carden, Province and Ferrebee ('40), excrete somewhere between 10 and 40% of the thiamine in their diet. They found an average excretion of 182 mg per day for twelve normal adults. Williams, Mason and Wilder ('43) found that when the intake of thiamine was approximately 1 mg per day, an excretion of 100 μg or more in 24 hours represented normal excretion.

After a few weeks on a diet containing 0.33 mg thiamine per 1000 cal., the subjects of an experiment conducted by Keys, Henschel, Mickelsen and Brozek ('43) excreted as an average about 10% of their intake in their urine. On the basis of physical tests, clinical observations and subjective reports, this intake was considered to be more than adequate. In this study the average thiamine intake per day was slightly lower than the recommendation of the Food and Nutrition Board ('45) for women weighing 56 kg and considerably lower than the recommended allowance corrected to the weights of the women (Kaucher, Moyer, Williams and Macy, '46), but the urine data presented indicate that these subjects were not thiamine deficient, although thiamine intakes were lower than the recommended allowances and the average thiamine to calorie ratio was 0.4 instead of 0.7. Holt

('44) believes that 0.24 to 0.44 mg thiamine per 1000 cal. is protective, and that the same values appear to be valid for all age groups and for pregnant women. His maximum value also seems adequate for lactating women in data presented here.

The diets during the study were high in fat and protein, therefore, one might expect the B_1 requirements to be somewhat lower than on a diet higher in carbohydrate.

Analyses showed 41% of the calories were contributed by fat, a percentage well above the 20 to 25% recommended by the Food and Nutrition Board. Non-fat calories were calculated from the percentage of fat in food composites which were analyzed after drying under vacuum from the frozen state (Flosdorf and Mudd, '38). The values were converted to calories with the factor 9.45. The non-fat calories for the mature milk periods averaged 59% of the total caloric intake. The average ratio of thiamine in the milk to non-fat calories in the diet was 0.76.

Fasting levels of thiamine in urine have been suggested by some investigators as a reliable index of thiamine status. Johnson, Sargent, Robinson and Consolazio ('45) of the Harvard Fatigue Laboratory have carried on extensive studies of the nutritional status of normal young men under various environmental conditions. Because there are conflicting evidences of deficiency and non-deficiency in the same individual when several biochemical tests are applied, they usually have attached significance to one test only when corroborated by the result of a second. For thiamine a fasting urinary excretion of less than 0.6 μg per hour and an excretion after a 5-mg test dose of less than 20 μg in 4 hours was considered to indicate an unsatisfactory thiamine status. Table 4 gives fasting urine values obtained from 1 hour samples collected on the first mornings of 5-day periods during which the women were studied and the mornings following the same 5-day periods. Thus, fasting samples were obtained which reflect the voluntary food intakes of the women before the experimental period and other samples portray the changes consequent to

ingesting the known diet for 5 days. All of the fasting excretions exceeded the 0.6 μg per hour minimum suggested by Johnson, Sargent, Robinson and Consolazio ('45).

Ten of the thirteen determinations before and after ingesting for 5 days a diet providing an average of 1.3 mg of thiamine per day show decreases in the rate of excretion in the urine, in two there were increases, and one pair of values indicate no change. While all of the values following

TABLE 4

Thiamine in fasting samples of urine at start and after 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	FIRST MORNING	AVERAGE DAILY INTAKE DURING 5 DAYS	FOLLOWING MORNING
	days	*μg/hr.*	*mg*	*μg/hr.*
J.M.	75–79	7.1	1.38	5.7
	173–177	8.4	1.59	7.7
V.K	95–99	12.8	1.07	8.6
	144–148	7.3	1.20	11.4
V.L.	68–72	6.9	1.50	4.8
	152–156	5.6	1.61	5.6
V.G.	78–82	11.3	1.36	5.4
	161–165	6.6	1.18	8.0
	239–243	9.7	1.18	6.0
	302–306	7.7	1.38	6.0
B.S.	85–89		1.11	4.8
	204–208	7.9	1.32	5.0
	259–263	8.8	1.26	3.6
V.S.	70–74	62.0	1.40	12.4

the 5-day period indicate that the thiamine intakes supplied all the needs of the lactating women's bodies, it seems likely that the intakes of ten of the women were higher before than during the 5-day period.

The excretion values for V.S. are of special interest. This subject was taking commercial vitamin supplements daily (6250 I.U. vitamin A, 3.6 mg of thiamine, 2.1 mg of riboflavin, vitamins D and E, niacin, pyridoxine and pantothenic acid) throughout pregnancy and during the interval between the

tenth day postpartum and the 5-day period during the third month of lactation. That she retained in her body some of this additional intake for more than a day is evidenced by the fact that the fasting urine sample on the first mornings of the period showed an excretion of 62.0 μg of thiamine per hour, a rate five to ten times greater than that of other subjects. Of the 3.99 mg excreted by V.S. during the 5-day period, 1.02 mg was excreted during the first 24 hours.

SUMMARY

The thiamine intake for 5-day periods, secretion in 24-hour collections of milk, and daily excretion in urine were determined for normal multiparas during the first 10 days postpartum and at various intervals in mature milk production. The intakes of the mothers were comparable, qualitatively, but quantity was determined by appetite. With an average intake of 1.1 mg thiamine per day, the average daily secretion in milk increased from 1 μg on the first day to 95 μg on the tenth day and the excretion in the urine ranged from 200 μg on the first day to 145 μg on the ninth day. During the period of highest secretion of thiamine in milk, 2–3 months postpartum, thiamine in the milk averaged only 8% of the intake.

The average volumes of the mature milk secreted during 5-day periods between 2 and 10 months postpartum, ranged from 268 to 1020 ml. The average daily thiamine content of the milk ranged from 30 to 162 mg. Of the thiamine intake accounted for during eighteen 5-day periods for ten women, averages of 23 and 8% appeared in the urine and milk, respectively.

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METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

IX. THE UTILIZATION OF RIBOFLAVIN DURING LACTATION [1]

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FOUR FIGURES

(Received for publication May 20, 1946)

Riboflavin is required in increased amounts for tissue regeneration and for accelerated metabolic activity (Copping, '45) and, although little evidence is available, it has been assumed that actively growing children and pregnant and lactating women have higher requirements than those of other adults (Copping, '45). Sure's ('40) experiments with rats have indicated that the riboflavin requirement for the rearing of young are at least five to six times that required for growth. The Food and Nutrition Board of the National Research Council ('45) has recommended riboflavin intakes per day of 2.0 mg for adult men and women, eating diets providing 3000 kg cal. per day, 2.5 mg for pregnant women, and 3.0 mg for lactating women. Keys and co-workers (Keys, Henschel, Mickelsen, Brozek and Crawford, '44; Keys, Henschel, Taylor, Mickelsen and Brozek, '44) have questioned the allowance

[1] The investigation represented in part by this paper was partially supported by a grant from The Nutrition Foundation, Inc., and was made possible by the cooperation of J. P. Pratt, M.D., Chief of the Department of Obstetrics, B. M. Hamil, M.D., Department of Pediatrics, Elizabeth Moran, R.N., Director of Nurses and Annie Lou Wertz, B.S., dietitian, all of the Henry Ford Hospital, Detroit.

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for normal adult men after studies of men receiving restricted riboflavin intakes. After 5 months on a diet providing 0.31 mg of riboflavin per 1000 cal (0.99 mg of riboflavin per day), no physiological handicaps were found in normal young men. Najjar, Johns, Medairy, Fleischmann and Holt ('44) studied the biosynthesis of riboflavin in man and concluded that riboflavin may not be a dietary essential under all conditions, since synthesis of riboflavin by the intestinal bacteria seemed to be an additional source of this vitamin. Recent work with rats, however, indicates that the absorption of riboflavin from the intestine probably is not an important source of this substance (Obermeyer, Wertz and Emerson, '45; Schweigert, McIntire, Henderson and Elvehjem, '45).

EXPERIMENTAL

During an investigation of the utilization of nutriments from food by lactating women, riboflavin was determined in the diets, milk and urine of normal lactating women.

The subjects of the investigation were multiparas with medical records of good or excellent health and successful nursing. Thirteen women participated in the study. Seven were studied during two consecutive 5-day periods immediately following delivery. Five were observed for 1 day 7 to 9 days postpartum. Nine of the thirteen mothers were studied during seventeen 5-day periods at various intervals during the production of mature milk. All of the diets were comparable, qualitatively, but the amounts of all foods except milk were varied so that the appetites of the women were satisfied. Duplicates of each meal during a 5-day period were composited for analysis. Complete 24-hour collections of milk and urine were obtained. Details of the method of manually expressing the milk and of collecting the food and milk are given in preceding publications (Kaucher, Moyer, Richards, Williams, Wertz and Macy, '45; Davies, '45). In conjunction with many periods during which mature milk was collected, fasting samples of urine were obtained the first morning of the 5-day period and the morning following the period. Each

collection represented excretion for 1 hour, 12 to 13 hours following the last meal.

Sample preparation and analysis

Food. About 400 gm of the ground 5-day food composite was homogenized in a Waring Blendor and the volume made to 500 ml. Of this, 20 ml was used for each of duplicate determinations. Each sample was diluted to 75 ml with water and enough 1 *N* sulfuric acid to make the mixture 0.1 *N* with respect to the acid and autoclaved for 10 minutes at 15 pounds pressure. When cool, the solution was adjusted to pH 4.5 with 2.5 *M* sodium acetate then incubated with clarase overnight (about 16 hours) at 37°C. with toluene. Finally, the sample was made to 100 ml, filtered, and 40 ml of the filtrate used for the riboflavin determination as described for milk (Roderuck, Coryell, Williams and Macy, '45).

Milk. An aliquot of the milk from each expression during a 24-hour period was poured immediately into a jar which was kept tightly covered in a refrigerator. The composite for the day preceding was taken to the laboratory each morning and held under refrigeration pending analysis by the method described in a preceding paper (Roderuck, Coryell, Williams and Macy, '45).

Urine. Collections of urine were combined, preserved with acetic acid (the final solution was 2% acetic acid) and kept under toluene until the 24-hour composite was complete. Each day the composite was taken to the laboratory, the volume checked and aliquots removed for analysis by the fluorometric procedure as described for milk (Roderuck, Coryell, Williams and Macy, '45). Filtration was the only pretreatment required with urine since all the riboflavin was in the free state, so measured amounts of filtered urine were added directly to Florisil columns. Usually 10 ml per liter of urine excreted was used for each determination.

RESULTS AND DISCUSSION

The average intake of riboflavin from the diets, analyzed "as eaten," was 3.1 mg per day, slightly more than the allow-

ance recommended by the Food and Nutrition Board of the National Research Council ('45) for a woman weighing 56 kg. Adjusted on the basis of the weights of the women studied the recommended allowance was 3.2 mg per day (Kaucher, Moyer, Williams and Macy, '46). The average intake of 3.1 mg was accomplished chiefly through two items in the diet: (a) there were large quantities of milk included in the daily menus, more than a quart and (b) liver was fed on the fifth day of each study period, thus increasing the average intake per day. The average of the analytical values for 5-day composites, 3.1 mg, was slightly less than the value of 3.3 mg calculated from tables of food composition (Kaucher, Moyer, Williams and Macy, '46). On the basis of the calculated figures, the average diet of the fifth day provided 51% more riboflavin than the mean intake for the other 4 days.

In table 1 are given the daily milk and urine volumes, the average riboflavin intakes, the daily secretions of total and free riboflavin in milk, and the daily excretions in the urine during the first 10 days postpartum. The averages of the values for each of the first 10 days postpartum are given in table 2.

Parallel with increasing milk production during the first 10 days postpartum, the secretion of riboflavin in the breast milk also increased. In general, riboflavin excretion in the urine also was augmented during the puerperium. Apparently, the diets eaten by the mothers provided for all the maternal needs following delivery and permitted increased production of milk containing equal or greater concentration of the vitamin. As recovery from childbirth and physiologic adjustment to lactation proceeded, greater excesses of intakes over requirements were portrayed by larger amounts in the urine. These contentions are supported by the effects of serving liver on the fifth and tenth days, which in the preceding paper (Roderuck, Coryell, Williams and Macy, '45), was noted to have a possible influence upon the mean concentration of riboflavin in milk on the fifth day and a greater effect on the tenth day. From the averages in table 2, the liver eaten on the

TABLE 1

Riboflavin intakes, excretion in urine and secretion in immature milk during first 10 days postpartum.[1]

SUBJECT	INTERVAL POSTPARTUM	VOLUME		RIBOFLAVIN			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	*ml*	*ml*	*mg*	*mg*	*mg*	*mg*
L.F.	1	71	2590	3.3	.02	.01	1.93
	2	200	1275	3.3	.08	.05	0.54
	3	733	2420	3.3	.33	.16	0.82
	4	1122	2075	3.3	.47	.22	1.09
	5	1441	1674	3.3	.53	.24	1.77
	6	1501	1718	3.3	.52	.25	1.85
	7	1596	1340	3.3	.62	.35	1.96
	8	1638	1810	3.3	.63	.35	2.34
	9	1676	1374	3.3	.65	.40	1.92
	10	1872	2220	3.3	.82	.52	3.32
V.G.	1	82	2067	2.6	.01	..	0.32
	2	177	1689	2.6	.03	.01	0.36
	3	849	1775	2.6	.23	.15	0.65
	4	1413	1323	2.6	.49	.32	0.75
	5	1471	1303	2.6	.55	.30	1.79
	6	1782	1148	2.2	.64	.39	1.56
	7	1630	1230	2.2	.63	.47	1.60
	8	1895	1237	2.2	.77	.57	1.70
	9	1828	1104	2.2	.74	.49	1.51
	10	1770	1075	2.2	.78	.47	1.96
V.K.	1	9	3086	2.4	..	..	1.07
	2	90	3651	2.4	.02	..	1.82
	3	484	2596	2.4	.10	..	2.51
	4	547	2427	2.4	.13	..	1.55
	5	560	2331	2.4	.16	..	2.09
	6	663	2249	2.6	.18	.13	2.08
	7	781	3380	2.6	.28	.18	2.11
	8	775	3840	2.6	.24	.17	1.98
	9	794	3115	2.6	.24	.17	1.92
	10	797	3328	2.6	.24	.17	2.44
V.L.	1	30	1757	3.2	..	..	0.87
	2	56	2686	3.2	..	..	0.83
	3	353	2759	3.2	.08	.05	0.80
	4	794	1823	3.2	.21	.16	0.84
	5	844	1847	3.2	.27	.18	2.22
	6	955	2710	3.6	.30	.21	2.10
	7	1047	2841	3.6	.30	.18	2.00
	8	1098	2066	3.6	.32	.20	1.81
	9	1118	2151	3.6	.31	.20	1.54
	10	1200	1669	3.6	.36	.22	2.78

TABLE 1 — (*continued*)

SUBJECT	INTERVAL POSTPARTUM	VOLUME		RIBOFLAVIN			
		Milk	Urine	Intake	Milk		Urine
					Total	Free	
	days	*ml*	*ml*	*mg*	*mg*	*mg*	*mg*
J.M.	1	35	2182	3.2	..	..	0.80
	2	385	1386	3.2	.08	.06	0.88
	3	870	1519	3.2	.27	.18	1.05
	4	1011	1201	3.2	.38	.18	1.00
	5	1121	1283	3.2	.48	.30	2.43
	6	1125	1172	3.3	.48	.32	1.90
	7	1287	1161	3.3	.54	.36	1.88
	8	1136	1520	3.3	.51	.42	1.94
	9	1258	....	3.3	.48	.35	...
	10	1336	1553	3.3	.65	.54	2.47
C.O.	1	16	1864	3.0	..	..	0.42
	2	100	939	3.0	.02	..	0.28
	3	335	1511	3.0	.07	.02	0.42
	4	595	....	3.0	.17	.09	...
	5	725	....	3.0	.23	.11	...
	6	821	....	2.8	.24	.15	...
	7	798	1470	2.8	.26	.17	1.46
	8	950	1127	2.8	.30	.17	1.36
	9	931	1191	2.8	.29	.16	1.34
	10	660	....	2.8	.24	.14	...
V.S.	1	6	1869	3.4	..	..	1.13
	2	92	1874	3.4	.02		0.77
	3	420	2887	3.4	.14	.08	1.15
	4	600	2063	3.4	.21	.11	1.32
	5	697	2065	3.4	.25	.15	2.48
	6	756	1502	3.2	.27	.17	2.05
	7	818	2524	3.2	.30	.18	2.57
	8	837	2237	3.2	.30	.19	2.17
	9	932	1984	3.2	.34	.24	2.26
	10	924	2287	3.2	.36	.23	3.46
M.B.	9	1189	1035	3.6	.49	..	1.67
E.L.	8	688	677	3.2	.28	..	0.65
M.S.	7	880	1355	3.4	.31	.24	2.07
G.S.	7	1017	1981	2.6	.33	.20	1.86
F.W.	7	953	1032	2.8	.37	.18	1.27

[1] The first day postpartum was variable to the extent of differences in the times at which the women delivered. For subjects delivered after 12 M. the first day began the following morning. The first day for C.O. was 22 hours; for L.F., 23.5 hours. V.G. was delivered 8 hours before the beginning of the first day postpartum; V.K. 17 hours; V.L., 5 hours; J.M. 16 hours; and V.S. 7 hours.

fifth day had only a slight effect in increasing the riboflavin contents of the milk, which were consistent with increases in milk volume. Although the average urine volume decreased on the fifth day, the average riboflavin content was more than doubled. From the fifth to ninth days, excretions decreased but ingestion of liver on the tenth day produced an excretion higher than that on the fifth day. Although the liver may have some effect on the riboflavin secretion on the tenth day, it

TABLE 2

Riboflavin in 24-hour collections of immature milk and urine during the first 10 days postpartum.

INTERVAL POSTPARTUM	MILK			URINE		
	Volume	Riboflavin		Volume	Riboflavin	
days	*ml*	*mg/day*	*mg/100 ml*	*ml*	*mg/day*	*mg/100 ml*
1	36	.01	.020	2202	0.93	.042
2	157	.04	.023	1928	0.78	.040
3	578	.18	.029	2210	1.06	.048
4	869	.30	.033	1819	1.09	.061
5	980	.35	.035	1750	2.13	.127
6	1086	.38	.034	1750	1.92	.119
7	1081	.40	.036	1831	1.88	.114
8	1127	.42	.037	1814	1.74	.106
9	1216	.44	.036	1708	1.74	.114
10	1223	.49	.039	2022	2.74	.147

seems that the amounts in human milk during the puerperium are determined principally by the physiologic processes operating during the development of milk flow and conform to the individual's needs for lactation.

Table 3 presents the average daily values for all of the 5-day periods included in the investigation. For all of the women studied, the volumes of milk secreted during the sixth to tenth day postpartum were more than double the amounts produced during the first 5 days and were larger than the amounts obtained from the same women during later lactation. The augmented milk flow during the puerperium was accompanied by corresponding increases in riboflavin. The relationship of volume and riboflavin content, for the same

TABLE 3

Average daily riboflavin intakes, excretion in urine and secretion in milk during thirty-two 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	VOLUME		INTAKE	RIBOFLAVIN					
					Milk				Urine	
					Total		Free			
	days	*ml*	*ml*	*mg*	*mg*	*% intake*	*mg*	*% intake*	*mg*	*% intake*
M.B.	72–76	718	691	2.7	.26	10	.15	6	0.69	26
L.F.	1–5	713	2007	3.3	.29	9	.14	4	1.23	37
	6–10	1657	1692	3.3	.65	20	.38	11	2.28	69
V.G.	1–5	798	1631	2.6	.26	10	.20[1]	8	0.78	30
	6–10	1781	1159	2.2	.72	32	.48	22	1.67	76
	78–82	848	1281	3.2	.41	13	.32	10	1.44	45
	161–165	901	993	3.2	.38	12	.26	8	1.17	36
	239–244	681	810	3.4	.29	9	.18	5	1.21	36
	302–306	394	1080	3.1	.18	6	.11	4	1.28	41
V.K.	1–5	338	2818	2.4	.08[1]	3	..	..	1.81	74
	6–10	762	3182	2.6	.24	9	.16	6	2.11	82
	95–99	647	2804	3.0	.24	8	.18[1]	6	1.82	60
	144–148	325	1447	3.1	.11	4	.07	2	1.22	40
V.L.	1–5	415	2174	3.2	..	..	.13[1]	4	1.11	35
	6–10	1084	2287	3.6	.32	9	.20	6	2.04	57
	68–72	789	2849	3.5	.25	7	.17	5	1.39	40
	152–156	680	1756	3.5	.22	6	.13	4	1.26	36
J.M.	1–5	684	1514	3.2	.24[1]	8	.18[1]	6	1.23	39
	6–10	1228	1352[1]	3.3	.53	16	.40	12	2.05[1]	62
	75–79	708	2265	3.4	.33	10	.27[1]	8	1.50	44
	173–177	268	2003	3.3	.11	3	.06	2	1.12	34
D.M.	45–49	401	1741	..	..	..	..	..	..	..
C.O.	1–5	354	1438[1]	3.0	.10[1]	3	.08[1]	2	0.37[1]	12
	6–10	832	1263[1]	2.8	.26	9	.15	6	1.39[1]	50
B.S.	85–89	1020	1347	3.1	.46	15	.32	10	1.33	43
	204–208	913	1356	3.3	.45	14	.37	11	1.80	55
	259–263	676	1219	3.2	.33	10	.25	8	1.75	54
M.S.	58–62	947	939	3.4	.36	11	.25	8	1.37	14
G.S.	80–84	899	1078	2.9	.41	14	.31	10	0.95[1]	32
V.S.	1–5	363	2152	3.4	.12[1]	4	.12[1]	3	1.37	40
	6–10	853	2107	3.2	.32	10	.20	6	2.50	77
	70–74	304	1852	3.3	.12	4	.08	2	2.05	61

[1] Less than five 24-hour collections included in average. For total riboflavin in milk, values were not obtained for first day but values calculated from the concentration in milk of second day are included in average (table 1).

women, continued through the periods of mature milk secretion. Both volume and riboflavin content varied widely for different individuals, but in general, larger secretions of riboflavin corresponded with large productions of milk.

The second 5-day study, or the period 6 to 10 days postpartum, is unusual in that the largest percentage of riboflavin is accounted for at that time. In the first 5 days, it is possible to account for less than 50% of the intake, perhaps owing to an extra demand for riboflavin in parturition. However, it is also possible that all of the riboflavin eaten as liver at noon on the fifth day was not excreted before the end of that day. The mechanism for such a lag in elimination has been suggested by Klein and Kohn ('40) who investigated the synthesis of flavin-adenine-dinucleotide from riboflavin by human blood cells. They found that synthesis occurred in vitro and in vivo. The concentration of dinucleotide in the cells was increased when supplements of riboflavin were fed, so they suggested that the ability of the cells to synthesize and store the dinucleotide might indicate that the cells serve to limit the loss of this vitamin by renal excretion. This may provide a partial explanation of the higher percentage of riboflavin accounted for in the second 5 days postpartum, the only 5-day period begun immediately following a day in which liver was known to be included in the diet.

The urine excretion during the mature milk study periods averaged 43% of the riboflavin intake, or 1.37 mg per day. In published studies of urinary excretion of riboflavin, wide variation is found among normal individuals on normal diets, but an excretion of 1.37 mg per day is in the highest range of the normal. Strong, Feeney, Moore and Parsons ('41) found that the normal excretion on unrestricted diets appeared to be of the order of 500 to 800 μg per day. This value decreased rapidly to from 50 to 150 μg on a dietary intake of from 1 to 2 mg of riboflavin. Since values of less than 200 μg per day are usually considered to indicate an unsatisfactory intake, he concluded such an intake was, at best, no more than marginal and perhaps insufficient. Axelrod, Spies and Elvehjem

('41), reported that two normal people excreted 236 and 270 μg per day. Williams, Mason, Cusick and Wilder ('43) studied two women and found that they excreted 1600 to 1800 μg per day on an intake of 3.6 mg per day. On an intake of 1.6 mg per day, they excreted 400 to 700 μg. Other data indicated that when the intake was about 1 mg, the excretion varied between 150 and 350 μg per day. From these data and other observations, they concluded that 1.6 mg of riboflavin per day (or 0.8 mg/1000 cal.) was not associated with tissue depletion, that 1 mg per day (0.5 mg/1000 cal.) depleted tissue stores only to a slight degree and 0.7 mg per day (0.35 mg/1000 cal.) unmistakably caused tissue depletion.

Ferrebee ('40) found that five normal subjects on their usual diet excreted 700 to 1700 μg per day. On regular hospital diets of 2400 cal., normal subjects excreted 1200 ± 200 μg for 5 days. Sebrell, Butler, Wooley and Isbell ('41) found that seven women on an institution diet excreted 130 to 810 μg per day, averaging 357. Ten subjects on a deficient diet (0.5 mg riboflavin per 2400 cal.) excreted from 30 to 168 μg per day, averaging 77 μg. Since close relationship between intake and excretion has been observed by all investigators, the excretion per day is not a satisfactory means of determining the riboflavin status of an individual. In our studies, again we have observed a direct relationship between intake and urinary excretion, especially the increased excretion of riboflavin on the day liver was included in the diet.

It is thought that the requirement for riboflavin is related to body size and weight as well as to the amount of food eaten. Riboflavin is known to exert a sparing action on thiamine (Elles and Zmachinsky, '37), and the relationship of thiamine to riboflavin has been used in making recommendations. The accepted ratio has been 1 to 1.5, or 0.7. That the women who participated in this investigation were in satisfactory thiamine status with average intakes lower than the recommended allowance has been pointed out (Roderuck, Williams and Macy, '46). The riboflavin intakes ranged from 2.2 to 3.6 mg

per day, averaging 3.1 mg, approximately the amount recommended by the Food and Nutrition Board of the National Research Council. The ratios of thiamine to riboflavin in the diets of the women ranged from 0.3 to 0.5, averaging 0.4, or 1 to 2.5. In figures 1 and 2 the thiamine and riboflavin contents of the 24-hour collections of both immature (fig. 1) and mature (fig. 2) human milk have been plotted against each other. The points for days on which the mothers ate liver are distinguished from other values. While the scatter diagram for the milk of the first 10 days shows a relationship between the amounts of thiamine and riboflavin secreted, the dispersion of the points during the period of rapidly increasing secretion portrays the variation among the individual subjects. For mature milk the diagram shows an almost perfect correlation between the two vitamins when the unusually high riboflavin values resulting from eating liver are disregarded. At all levels of mature milk secretion the average ratio between thiamine and riboflavin is approximately 0.4 (1 to 2.5), the same ratio as the average in the intake.

The ratios of thiamine to riboflavin plotted according to interval postpartum also are shown in figures 3 and 4. During puerperium the ratios for the first 5 days postpartum are low, showing daily secretions of thiamine in the milk to be less than one-tenth of the amounts of riboflavin in the same samples. During the fifth to tenth days postpartum the ratios increased, reaching the proportion of approximately 1 to 4 on the tenth day. Between the tenth and fiftieth days the ratios of thiamine to riboflavin reached the mature level. The ratios for mature milk, show the distribution of the average ratio of 1:2.5 for thiamine to riboflavin. The scatter is a consequence of variations in the composition of the milk of the different women, rather than day-to-day variations in milk of the same women, for in general, the values for the 5 consecutive days in each period for the same women tend to be grouped together.

Fasting sample values, calculated as excretion per hour, are of some significance. Johnson, Sargent, Robinson and

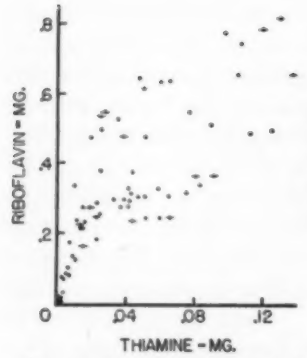

Fig. 1 Secretion of riboflavin and thiamine in immature human milk.

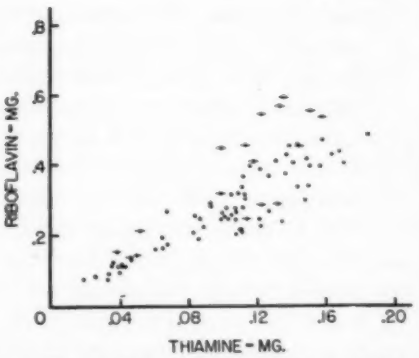

Fig. 2 Secretion of riboflavin and thiamine in mature human milk.

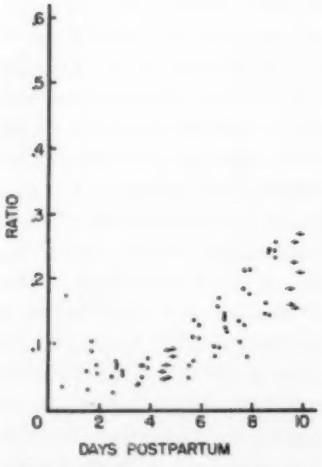

Fig. 3 Thiamine to riboflavin ratio in immature human milk.

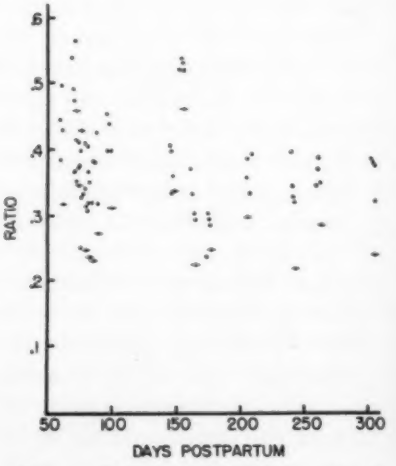

Fig. 4 Thiamine to riboflavin ratio in mature human milk.

Consolazio ('45), consider that a fasting urinary excretion of less than 20 μg of riboflavin per hour and an excretion after a test dose (5 mg of riboflavin) of less than 200 μg in 4 hours is significant and indicates a deficiency state.

Fasting samples were obtained on the first mornings of thirteen 5-day periods of study with lactating women and on the mornings following the experimental period (table 4). Of the fasting samples before the 5-day period, four were less

TABLE 4

Riboflavin in fasting samples of urine before and after 5-day periods.

SUBJECT	INTERVAL POSTPARTUM	FIRST MORNING	AVERAGE DAILY INTAKE DURING 5 DAYS	FOLLOWING MORNING
	days	*μg/hr.*	*mg*	*μg/hr.*
J.M.	75–79	54	3.4	66
	173–177	52	3.3	72
V.K.	95–99	47	3.0	68
	144–148	17	3.1	70
V.L.	68–72	19	3.5	47
	152–156	26	3.5	41
V.G.	78–82	38	3.2	36
	161–165	15	3.2	42
	239–243	20	3.4	30
	302–306	14	3.1	34
B.S.	85–89	..	3.1	59
	204–208	32	3.3	49
	259–263	22	3.2	25
V.S.	70–74	154	3.3	49

than 20 μg per hour. The second fasting samples were always taken less than 24 hours after liver had been served, and demonstrate the prolonged effect of the greatly increased intake on the day preceding. In control determinations with normal adults, fasting samples collected 12 hours after a meal containing liver were considerably higher than samples collected 12 hours after a meal containing smaller amounts of riboflavin. For V.S. the fasting excretion preceding the 5-day period was exceptionally high owing to the fact that she had

been taking a riboflavin supplement daily preceding the determination. Since other criteria indicate satisfactory nutritional status with respect to riboflavin for the mothers, it seems likely that fasting excretions of less than 20 μg per hour may be merely effects of low intakes during the preceding 24 hours, rather than evidence of deficiency. It may be that a fasting level of 20 μg per hour is a conservative estimate (may be somewhat too high).

Oldham, Lounds and Porter ('46) have presented evidence that urinary riboflavin excretions vary inversely with the nitrogen intakes, however, no relationshhip could be found for the mothers who collaborated in this study. Actually all of the subjects were in negative nitrogen balance during the period of 6 to 10 days postpartum, the period of greatest riboflavin excretion. Earlier work (Hunscher, Donelson, Nims, Kenyon and Macy, '33), from this laboratory showed that there is a tendency for lactating women to be in negative nitrogen balance for several weeks after parturition.

In studies of the urinary excretion of riboflavin, wide variation has been found among healthy individuals eating good diets. It is true in general, that only a fraction of the intake is excreted, but increased intake does lead to increased excretion and vice versa. For this reason the 24-hour sample has been concluded by some workers to be of little significance except as indication of the immediate intake, so fasting urine determinations have been investigated by several workers. Feder, Lewis and Alden ('44) believed that the concentration of riboflavin per milliliter of urine was a more constant figure than any other and on the basis of 800 μg per day being the average excretion, they calculated that the range per milliliter should be 0.53 to 0.8 μg. To eliminate boderline cases, less than 0.3 μg/ml was considered indicative of riboflavin deficiency. The concentrations per milliliter in the present study all exceeded this amount, frequently by very large margins.

The relationship of riboflavin to other dietary factors and to certain physical factors are frequently considered pertinent to the intake of this vitamin. In this study the average value

of 1.1 mg of riboflavin per 1000 cal. exceeds the 0.5 mg per 1000 cal. set by Williams, Mason, Cusick and Wilder ('43) as the minimum daily requirement and also exceeds the value 0.8 mg per 1000 cal. which they found was not associated with any tissue depletion. Too, the subjects' intakes averaged 0.053 mg of riboflavin per kg of body weight, which is substantially higher than the 0.025 mg per kg suggested as a minimum intake by Copping ('45) and also exceeds the 0.035 mg per kg considered minimal by Sebrell, Butler, Wooley and Isbell ('41). The estimated riboflavin intake per square meter of surface area (Boothby and Sandiford, '29) was 1.94 mg.

SUMMARY

Riboflavin was determined in composites exactly representing the food intakes of healthy nursing mothers during 5-day periods and in 24-hour collections of their milk and urine during the same intervals postpartum. The subjects were multiparas and received diets of comparable quality in amounts which satisfied their appetites.

The average daily intake of riboflavin was 3.1 mg per day. During the first 10 days postpartum, riboflavin secretion in milk increased with milk production, from an average of 0.01 to 0.49 mg per day. For the same women, the relationship of volume and riboflavin content continued during the periods of mature milk production. The average daily riboflavin secretion in mature milk was 0.29 mg.

While riboflavin secretion paralleled milk volume, the inclusion of liver in the diet of the fifth day of each 5-day period provided an intake of riboflavin 50% greater than the mean intakes for the other 4 days which produced only slight increases in secretion and excretion on the fifth day postpartum but augmented the amounts of riboflavin in milk and urine collected on the fifth day of all other 5-day periods.

While 3 to 32% of the average riboflavin intakes were found in the immature milk (first 10 days) and 3 to 15% of the intakes was secreted in mature milk, the excretion in urine during respective periods ranged from 12 to 82 and 26 to 61%

of the intake. Together the milk and urine accounted for 15 to 108% of the intake.

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SELF SELECTION OF DIET

III. APPETITES FOR B VITAMINS [1]

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(Received for publication June 12, 1946)

In 1933, Harris, Clay, Hargreaves and Ward showed that thiamine-deficient rats developed an appetite for foods containing thiamine, while normal animals showed no such appetite. They were able to produce evidence which clearly indicated that the thiamine appetite was developed because the animals learned that the thiamine-containing diet gave them a beneficial effect or "made them feel better." Later, Richter, Holt and Barelare ('37) described a craving of thiamine-deficient rats for thiamine solutions or yeast, and a less marked craving of riboflavin-deficient rats for riboflavin. Jukes ('38) could not demonstrate an appetite for riboflavin in riboflavin-deficient chickens. Richter and Hawkes ('41) recorded the voluntary intakes by normal rats of thiamine, riboflavin, nicotinic acid, and pyridoxine solutions. The amounts selected were far higher than the requirement — between 0.1–1.20 mg per day.

The present experiments were designed to determine if appetites for thiamine, riboflavin, pyridoxine, and pantothenate could be demonstrated in rats.

METHODS

In each experiment twenty rats were weaned at 21–25 days of age and divided into two groups of five males and five fe-

[1] Contribution no. 602 from the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

males each. Each animal was placed in a cage with a rack containing two cups. During a 3-week control period, one group had both cups filled with a vitamin-containing diet, while the other group had both cups filled with the standard diet.[2] Vitamins other than the one under study were supplied as pills.[3] The cups were alternated in a predetermined random manner, and the amount of food eaten from each cup was recorded daily. During the 3-week experimental period, one of the cups contained the standard diet and the other the vitamin-containing diet. The cups were interchanged in the same random manner as before and the amount of food eaten from each recorded daily. The method of presentation and statistical treatment of the data here reported was previously discussed (Scott and Quint, '46).

RESULTS

The results of the thiamine, riboflavin, pyridoxine and pantothenic acid experiments are recorded in table 1. The thiamine observations confirm the conclusions of Harris et al. All the thiamine-deficient animals began eating the thiamine-containing diet almost exclusively on the first or second day (average 1.3 days) and continued to do so until the sixth to twenty-first days (average 12.2 days). After this they ate from both diets.

With respect to the riboflavin experiment, only six out of ten riboflavin-deficient rats ate the riboflavin diet exclusively. They began to do so on the first to thirteenth day (average

[2] The standard diet consisted of 24% purified casein (Labco "Vitamin-Free"), 10% hydrogenated fat (Primex), 4% salts (Jones and Foster, '42), and 62% sucrose. The vitamin-containing diets had in addition: Thiamine diet, 5 μg/gm thiamine hydrochloride; riboflavin diet, 5 μg/gm riboflavin; pyridoxine diet, 5 μg/gm pyridoxine hydrochloride; pantothenate diet, 10 μg/gm calcium pantothenate.

[3] Each pill contained approximately: 60 μg thiamine hydrochloride, 120 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 10 mg choline chloride, 1 mg α-tocopherol, and 55 I.U. Vitamin A and 11 I.U. Vitamin D, as 0.001 ml Natola; all in a dextrin-powdered sugar base. One pill was offered each animal daily. In the thiamine experiment, no thiamine was included in the pills; in the riboflavin experiment, no riboflavin was included, etc.

TABLE 1

Appetite for thiamine, riboflavin, pyridoxine or pantothenate acid. All data in terms of mean and standard error of the mean.

	GROUP A		GROUP B		DIFFERENCE (IN PER CENT EATEN) ATTRIBUTABLE TO THE VITAMIN DEFICIENCY (A–B)
	Control period	Experimental period	Control period	Experimental period	
Diet	*Standard*	*Choice*	*Thiamine*	*Choice*	
Wt. gain (gm)	15.6 ± 2.2	77.3 ± 4.5	63.9 ± 4.6	39.3 ± 6.3	
Total food eaten (gm)	94.0 ± 4.6	189.2 ± 7.6	155.2 ± 9.8	199.1 ± 14.5	
Average *thiamine* intake (μg/day)	0	36.0	37.0	27.3	
Change in per cent eaten from cup 1[1] (control minus experimental)		29.8 ± 5.2		8.8 ± 6.9	21.0 ± 8.5
Diet	*Standard*	*Choice*	*Riboflavin*	*Choice*	
Wt. gain (gm)	21.6 ± 3.0	73.5 ± 8.3	85.9 ± 3.1	59.9 ± 7.1	
Total food eaten (gm)	96.4 ± 3.0	210.4 ± 10.4	174.3 ± 10.8	262.5 ± 13.4	
Average *riboflavin* intake (μg/day)	0	34.0	41.5	34.3	
Change in per cent eaten from cup 1[1] (control minus experimental)		26.3 ± 11.2		1.8 ± 4.5	24.5 ± 12.1
Diet	*Standard*	*Choice*	*Pyridoxine*	*Choice*	
Wt. gain (gm)	32.2 ± 2.0	75.7 ± 3.0	77.0 ± 2.5	62.8 ± 4.2	
Total food eaten (gm)	105.7 ± 3.7	222.1 ± 7.6	172.8 ± 5.3	267.7 ± 7.0	
Average *pyridoxine* intake (μg/day)	0	41.9	41.1	36.2	
Change in per cent eaten from cup 1[1] (control minus experimental)		30.9 ± 7.5		8.2 ± 3.6	22.7 ± 8.1
Diet	*Standard*	*Choice*	*Pantothenate*	*Choice*	
Wt. gain (gm)	25.3 ± 2.1	42.0 ± 4.5	68.6 ± 3.3	60.9 ± 6.4	
Total food eaten (gm)	120.6 ± 8.6	156.3 ± 10.7	169.7 ± 8.2	237.6 ± 13.3	
Average *pantothenate* intake (μg/day)	0	37.6	80.8	59.7	
Change in per cent eaten from cup 1[1] (control minus experimental)		− 2.8 ± 7.0		3.0 ± 10.7	− 5.8 ± 12.8

[1] Cup 1 contained the standard diet during the experimental period; a positive change in per cent eaten (control minus experimental) is therefore evidence of appetite for thiamine.

4.2 days) and continued until the fourth to twenty-first day (average 15.0 days). In the experiment with pyridoxine eight of ten deficient animals ate the pyridoxine-containing diet starting on the first to fifth days (average 2.4 days) and continued until the fourth to twenty-first days (average 13.9 days). In all three cases, there can be no doubt that an appetite for the vitamin was shown.

In three experiments on calcium pantothenate no appetite for this substance could be detected. The data yielded by one of these experiments are presented in table 1. The results of other experiments in which the choices contained 0 and 5 μg/gm and 2 and 10 μg/gm of pantothenate were similar. An experiment outlined in table 2 gave a more definite indication of an

TABLE 2

Effect of flavor on pantothenate appetite.

All data in terms of mean and standard error of the mean.

	GROUP A		GROUP B		DIFFERENCE (IN PER CENT EATEN) ATTRIBUTABLE TO PANTOTHENATE DEFICIENCY (A–B)
	Control period	Experimental period	Control period	Experimental period	
Diet	*Standard or standard plus anise*	*Choice* [1]	*Pantothenate or pantothenate plus anise*	*Choice* [1]	
Wt. gain (gm)	29.9 ± 3.4	56.3 ± 10.4	69.1 ± 3.8	64.9 ± 6.2	
Total food eaten (gm)	98.5 ± 5.2	179.2 ± 17.8	158.3 ± 8.1	255.5 ± 8.9	
Average *pantothenate* intake (μg/day)	0	60.9	75.4	58.6	
Change in per cent eaten from cup 1 (control minus experimental)		23.6 ± 16.0		— 4.4 ± 3.3	28.0 ± 16.3

[1] Half of the animals in each group were given their choice of unflavored standard diet and flavored pantothenate diet; the other half chose between flavored standard diet and unflavored pantothenate diet.

appetite. Here one of the choices was labeled with anise flavor (10 p.p.m.) which a previous experiment has shown is itself neither liked nor disliked by rats (Scott and Quint, '46). In the control period, one-fourth of the animals were fed the standard diet, one-fourth the anise-flavored standard diet, one-fourth the pantothenate (10 μg/gm) diet, and the other fourth a flavored pantothenate diet. The choices during the experimental period are described in the footnote to table 2. During this latter period seven of the ten pantothenate-deficient animals ate almost exclusively the pantothenate diet; the other three selected just as definitely the standard diet. Five out of the ten ate a flavored diet. Of the ten normal animals in Group B, none made a definite choice.

DISCUSSION

An appetite as shown in a self-selection experiment may be classed as one of the following types, depending on its origin: (1) simple preferences, (2) learned appetites, or (3) true hungers. The first type is distinguishable because simple preferences have no relation to nutritional value, and are due to flavor, odor, consistency, or some similar quality of the food. Learned appetites are based upon the ability of the animal to learn by experience that a certain food will give him a feeling of well-being. Hungers are appetites that are based directly on physiologic need and require no learning process. The appetite for calories, previously discussed (Scott, '46), could scarcely be other than a true hunger. Richter ('42) and Young ('41) consider all appetites for dietary essentials as being true hungers.

The present experiments show that appetites for foods containing thiamine, riboflavin, or pyridoxine are developed in rats whose diets have been deficient, respectively, in these vitamins. These appetites are not simple preferences, because they cannot be found in normal animals. They must therefore be either learned appetites or hungers. If a difference in nutritional need is accompanied by a difference in appetite, then the appetite is related to the need, but since

hungers are related directly and learned appetites are related indirectly to need, the evidence here reported is not conclusive in deciding which type of appetite is involved in the preferences for foods containing these vitamins. The fact that the appetites are not developed by all animals and are not developed immediately may be considered, however, as limited evidence that the appetites are learned.

The lack of appetite for pantothenate under the same circumstances may be due either to an insufficient stimulus, i.e., incapacity of the animal to appreciate the beneficial effect of this vitamin, or to inability to distinguish the two diets, or to both factors. The previously described repetitious character of eating behavior when diets are identical indicates that identification of a particular diet is not difficult for a rat, and indeed in the case of thiamine, riboflavin and pyridoxine, the fact that repetitious eating occurs allows the possibility that identification of the beneficial diet may have occurred without sensory perception of the vitamin itself. While it is true that flavoring necessarily makes identification easier and thus could encourage repetitious eating, this fact in itself does not prove that inability to distinguish the two diets was the cause of the failure to demonstrate a pantothenate appetite when no flavor was added, for it would seem equally reasonable that a weaker stimulus would require a more positive identification for choice to be made. The stimulus provided by the beneficial effect of pantothenate cannot be altogether lacking because in such case no preference for foods containing pantothenate would occur even when the choices were clearly distinguishable by flavor.

In any case, the pantothenate appetite as demonstrated must be a learned appetite, since it is associated with a flavor or lack of flavor and not with the vitamin itself. The animals must therefore learn what flavor is to be associated with the vitamin and its beneficial effect, while true hungers should not require a learning process. The experiment on flavor and pantothenate confirms the previous observation that rats have no preference for nor dislike of anise.

SUMMARY

In the cases of thiamine, riboflavin, and pyridoxine, an appetite for foods containing the vitamin is developed in animals previously fed a diet deficient in that vitamin. Normal animals do not show such appetites. Appetites for pantothenate cannot be developed in pantothenate-deficient animals unless the choices are labeled with a flavor, and even in this case some animals make the wrong choice. It is concluded that appetite for foods containing pantothenate is a learned appetite and not a true hunger.

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SELF SELECTION OF DIET

IV. APPETITE FOR PROTEIN [1]

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TWO FIGURES

(Received for publication June 12, 1946)

In a recent study (Scott, '46) it was found that a considerable percentage of rats failed to eat casein, when the dietary choices offered were this protein, sucrose, hydrogenated fat, and a salt mixture. The present studies were undertaken to obtain further evidence on the nature of this failure to eat an essential nutrient.

METHODS

In each of the first three experiments, ten male and ten female rats were weaned at 21 to 25 days of age and placed in individual cages with four food cups all filled with a mixed diet.[2] The experiments differed only in that different proteins — lactalbumen, fibrin, or egg albumen [3] — were used in the diet. Vitamins were given separately as pills.[4] During

[1] Contribution no. 607 from the Department of Chemistry, University of Pittsburgh. Aided by grants of the Nutrition Foundation, Inc., and the Buhl Foundation.

[2] The mixed diet consisted of 62% sucrose, 10% hydrogenated fat (Primex), 4% salts (Jones and Foster, '42), and 24% protein.

[3] Wilson's fibrin and Borden's lactalbumen were used. The egg albumen was prepared by dissolving commercial egg white, denaturing it in an autoclave, and extracting the product before drying with distilled water, alcohol, and ether successively.

[4] Each pill contained approximately: 60 μg thiamine hydrochloride, 120 μg riboflavin, 90 μg pyridoxine hydrochloride; 150 μg calcium pantothenate; 10 mg choline chloride; 1 mg α-tocopherol; and 55 I.U. Vitamin A and 11 I.U. Vitamin D as 0.001 ml Natola; all in a dextrin-powdered sugar base. One pill was given each rat daily.

a 3-week control period, the amount eaten from each cup was recorded daily and the cups were then interchanged. In a 3-week experimental period, the choices given the animals were sucrose, fat, salt mixture, and the same protein as in the control period, each in a separate cup. The amount eaten of each was recorded and the cups interchanged daily as before. Since it has been shown that the eating behavior of a rat tends to be like that of its littermates, no more than two animals in any experiment were taken from one litter.

In the fourth experiment, twenty males and twenty females were divided into four groups so that each animal had one littermate of the same sex in each of the other three groups. The members of each group were fed one of the following mixed diets in all of four cups during the control period: (1) 24% casein diet; (2) 24% lactalbumen diet; (3) 24% fibrin diet; (4) 24% egg albumen diet. In the experimental period all animals were given their choice in separate cups of these four mixed diets.

In the final experiment, each of thirty rats was placed in a cage with six cups all filled with a diet which consisted of 62% sucrose, 10% hydrogenated fat, 4% salt mixture, and 6% each of casein, fibrin, lactalbumen, and egg albumen. During the 3-week control period, the eating behavior of the rats was observed and recorded, and the cups interchanged as in previous experiments. In the experimental period seven choices, each in a separate cup, were offered the rats. These included sucrose, hydrogenated fat, casein, fibrin, lactalbumen, egg albumen, and salts. Since the amount of salts eaten has been found to be of a different order of magnitude than the other choices, in this case it was not interchanged with the other choices, but offered separately. The other choices were interchanged in the same random manner as during the control period.

RESULTS

The data on eating behavior obtained in the control period of the first four experiments were comparable to those previously presented. It was found that some difference in

growth rate could be observed when different protein sources were consumed (table 1) and the littermate data of the fourth experiment (table 2) showed that this was in part of nutritional origin. The position eating of the animals in the fifth experiment is of some interest. From the left to the right cups, the animals ate the following average per cent of their total food: 18.6, 15. 6, 13.3, 13.2, 16.1, 23.2.

TABLE 1

Growth rate and food consumption during the control period.

EXPT.	PROTEIN SOURCE	MALES			FEMALES		
		No. of animals	Growth	Food eaten	No. of animals	Growth	Food eaten
			gm	*gm*		*gm*	*gm*
1	Lactalbumen	10	85.0 ± 4.7	169.1 ± 10.1	10	75.3 ± 3.2	139.8 ± 5.3
2	Fibrin	10	70.4 ± 6.2	145.3 ± 10.7	10	69.3 ± 2.2	131.4 ± 7.1
3	Egg albumen	10	75.1 ± 4.1	152.7 ± 6.7	10	65.0 ± 2.8	145.9 ± 7.1
5	Mixture	15	94.1 ± 3.1	198.6 ± 4.9	15	78.5 ± 1.8	187.2 ± 4.6

[1] All data in terms of mean and standard error of the mean.

TABLE 2

Growth rate and food consumption during the control period. (Experiment 4)

PROTEIN SOURCE	NO. OF ANIMALS	GROWTH	FOOD EATEN
		gm	*gm*
Casein	10	76.5 ± 4.7	152.9 ± 11.5
Lactalbumen	10	86.3 ± 4.6	156.2 ± 9.7
Fibrin	10	87.5 ± 3.5	165.0 ± 8.0
Egg albumen	10	77.3 ± 4.4	158.1 ± 7.7

The results of the experimental periods of the first three experiments and of the seven choice experiments are presented graphically in figure 1. The choices of proteins of the rats in the seven choice experiments are presented in figure 2. Finally, the results of the experiment where a mixed diet was offered are shown in table 3.

TABLE 3

Appetites for mixed diets.

PROTEIN SOURCE IN CONTROL PERIOD	WT. GAIN	TOTAL FOOD	APPETITE FOR DIET CONTAINING [1]			
			Casein	Lactalbumen	Fibrin	Egg albumen
	gm	*gm*				
Casein	85.0±8.3	272.6±15.7	— 4.2±6.1	13.2±10.4	10.4± 9.4	—19.5±2.8
Lactalbumen	79.7±9.5	266.8±19.6	9.0±7.7	8.2± 9.5	1.7± 8.2	—18.9±3.8
Fibrin	76.5±8.0	275.8±15.2	12.2±5.6	0.6±13.0	3.5±11.2	— 9.2±3.2
Egg albumen	85.0±8.1	266.1±16.0	25.1±8.1	—18.3±12.0	13.3± 8.6	—20.2±8.5
Average	81.6±4.1	270.4± 8.0	10.9±3.7	0.9± 5.4	5.4± 4.7	—16.9±2.6

[1] Change in per cent eaten (experimental minus control) from corresponding cup during control and experimental periods.

DISCUSSION

If the animals in the first three experiments were divided into those which lost or gained weight during the experimental period (Groups A and B, respectively) only the results of the experiment on egg albumen and the seven-choice experiment differed significantly from the previous casein experiment. This is shown in table 4, where the distribution into groups A and B of the rats in each experiment and the probability of such distribution being due to random sampling is shown.[5] These probability considerations indicate that egg albumen

TABLE 4

Group distribution of animals on self selection.

PROTEIN CHOICE	NUMBER OF ANIMALS IN		PROBABILITY [2]
	Group A [1]	Group B	
Casein	34	53	1.000
Lactalbumen	9	11	.774
Fibrin	7	13	.408
Egg albumen	14	6	.006
All four	2	20	.010

[1] Group A lost weight during the experimental period; group B did not.

[2] Probability that a group distribution as improbable as that shown could arise solely through random sampling.

was avoided more than the other proteins and that in the seven-choice experiments, a large majority of animals found at least one protein they liked.

The differences in weight change and total calories between the data shown in figure 1 and the previous results on casein can be explained in terms of the difference in number of animals selecting protein, and of the greater growth of the animals during the control period in the later experiments. The only result in figure 1 which has no apparent explanation

[5] Probability was calculated by the method described in a previous paper (Scott, '46). The formula used in that paper was incorrectly stated and should have read

$$P_A = \frac{n' \cdot}{(n-x) \cdot n'} (.39)^x (.61)^{n-x}.$$

is the tendency of the rats in the fibrin experiment to eat less fat and more carbohydrate than the average.

The reason for the dislike of egg albumen in these experiments was not apparent, although this protein did differ in not being quite so finely ground as the others and it was more nearly tasteless. The avoidance of fibrin in the seven-choice and its acceptance in the four-choice experiment appears inexplicable.

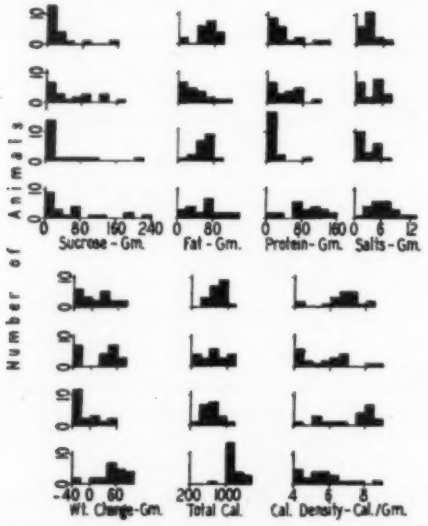

Fig. 1 Weight change and selections of animals when various proteins are offered.

The protein offered was in each case: Top histogram — lactalbumen; second histogram — fibrin; third histogram — egg albumen; lower histogram — casein, lactalbumen, fibrin, and egg albumen simultaneously.

A general principle of self selection may be stated as follows: If N choices are offered a group of animals, the number of possible preferential appetites that can be shown is N minus one. This is true because every dislike leads to an apparent appetite for the other choices, and conversely, each appetite appears to cause a dislike of the other choices. This is well

illustrated in the experiment on mixed diets, where table 3 indicates an apparent appetite for the diet containing casein. But if the dislike of the diet containing egg albumen is real, there should be an apparent average preference for each of the other three diets amounting to $16.9/3 = 5.6$ change in per cent eaten. If 5.6 is subtracted from the ostensible change of 10.9 in per cent eaten of casein, the latter change is no longer significant. If, on the other hand, the preference for the casein diet is considered real, a similar calculation indicates that the dislike of the egg albumen diet is still definitely significant. Thus only a dislike of the diet containing egg albumen was proved in the mixed diet experiment. Furthermore, there appeared to be no general effect of previous diet on appetite.

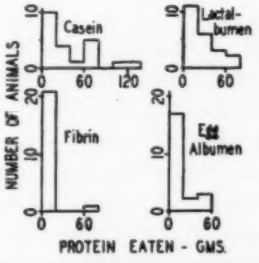

Fig. 2 Selection of proteins when offered simultaneously.

It may be asked whether an appetite for proteins in general exists in the rat, or whether the appetites for casein, lactalbumen, fibrin, and egg albumen are independent of each other. The seven choice experiment seems to indicate the latter is true, and that animals which dislike casein do not necessarily dislike all proteins. The validity of this conclusion was tested as follows: The probability that a rat would not eat casein, lactalbumen, fibrin, or egg albumen when offered individually was 0.39, 0.45, 0.35, and 0.70 as determined experimentally. If these probabilities are independent, the probability that an animal would not eat all four is the product of the individual probabilities which equals 0.043. Accepting the validity of this figure, the probability of occurrence in a random

sample of a distribution such that two out of twenty-two rats would not eat protein is 0.241. Thus the evidence from these experiments must be interpreted as indicating that appetites for different proteins are largely independent.

In a previous discussion (Scott and Quint, '46) it was pointed out that appetites as shown by self selection can be classified into three types depending on their origin. These types were called simple preferences, learned appetites, and hungers. Since there appears to be no appetite for protein in the general sense, and since the nutritional need for protein bears a poor relation to selection of protein, the preferences shown by some animals for various proteins cannot be considered as a true hunger. They may be simple preferences, or they may be learned appetites if one assumes that certain animals are unable to learn to associate beneficial effects with protein. But since it was shown that appetites for different proteins must be essentially independent, while the beneficial effects of the proteins used were approximately equal, a different type of learning, or a learning process dependent on different trivial factors, must be assumed for each individual protein, if a learned appetite is involved. Under these circumstances, the more obvious conclusion that the appetites for various proteins are simple preferences, appears probable.

SUMMARY

When rats were offered their choice of sucrose, hydrogenated fat, salt mixture, and a protein, approximately the same proportion of animals refused to eat the protein if it was casein, lactalbumen, or fibrin. If it was egg albumen, many more refused. If these four proteins were offered simultaneously as choices, only two out of twenty-two animals refused to eat at least one of them. If the choices were offered as part of mixed diets, the animals avoided the diet containing egg albumen, but showed no other marked preferences. It was concluded that the appetites for different proteins were largely independent and that no "appetite for protein" in

the general sense was indicated. The appetite for various proteins, found in some animals, is apparently based on simple preference, although it may possibly be a learned appetite.

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PROTEIN CONCENTRATES AS AMINO ACID SOURCES FOR THE CHICK: CORN GLUTEN MEAL, COTTONSEED MEAL AND PEANUT MEAL

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(Received for publication, April 9, 1946)

It has recently become possible to compound a diet of purified or synthetic ingredients in which a particular protein concentrate is the sole source of amino acids for the chick. Such a diet has been used in this laboratory to study the proteins of soybeans (Almquist et al., '42), beef blood (Grau and Almquist, '44a), sesame seeds (Grau and Almquist, '44b) and sunflower seeds (Grau and Almquist, '45b). The present report, which is a continuation of these studies, is concerned with the proteins of corn gluten meal, cottonseed meal and peanut meal.

The ideal way to evaluate proteins as amino acid sources would be to compare the chick's requirement for a particular amino acid with the chemical analysis for that acid in a protein. However, some of the amino acid requirements are still unknown, and the analytical data for the various acids are not all of equal accuracy. Hence feeding trials in which amino acids are used as supplements are essential for the evaluation of protein concentrates.

The literature on the amino acid composition of proteins has been summarized up to 1945 by Block and Bolling ('45). These data, together with some obtained in this laboratory for tryptophane (Kratzer, '44) and methionine (Grau and Alm-

quist, '45a) have served as bases for comparison with the known amino acid requirements (Almquist, '45).

METHODS

White Leghorn chicks which were reared on a commercial-type mash for the first 2 weeks after hatching were weighed every other day during the second week. On the basis of weight and rate of gain, groups of 6 to 12 chicks were selected and given the experimental diets. The birds were housed in electrically heated battery brooders with wire floors; feed and water were supplied ad libitum. The chicks and feed were weighed every other day during an 8-day period. The basal diet consisted of calcium gluconate 8, tricalcium phosphate 2, dipotassium phosphate 0.5, potassium chloride 0.3, sodium chloride 1.0, choline chloride [1] 0.2, inositol 0.1, cholic acid 0.1, crude soybean oil 5, mixed tocopherols [2] 0.05, and fortified sardine oil, 3000 A–400 D per gm) 0.25 gm per 100 gm diet, and thiamine 1, riboflavin 1, pyridoxine 1, nicotinic acid 3, calcium (d) pantothenate 3, 2-methyl, 1–4, naphthohydroquinone diacetate 1, biotin [3] 0.1, folic acid [4] 1, manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8 and cobalt 0.5 mg per 100 gm diet. Enough of the protein concentrate was added to provide 20 gm of crude protein in the diet, and glucose [5] was added to a total of 100 gm. The various supplements replaced glucose in the diet. The corn gluten meal contained 45.6% crude protein (N $\times$ 6.25), the cottonseed meal 44.4% and the peanut meal 42.7%.

The growth rates were obtained by multiplying the average gain per day by 100 and dividing by the average weight during

[1] Choline chloride was provided by Lederle Laboratories, Inc., through the courtesy of Dr. T. H. Jukes.

[2] Concentrate of natural mixed tocopherols (15%), Distillation Products, Inc.

[3] Biotin was provided by Merck and Co., through the courtesy of Dr. J. C. Keresteszy.

[4] Folic acid was provided by Lederle Laboratories, Inc., through the courtesy of Dr. E. L. R. Stokstad.

[5] Cerelose.

the experiment, to give the per cent gain per day (Grau and Almquist, '43). The results of supplementation on growth were usually very marked, so that there was seldom any doubt of the significance of the results. Since it was found that when the experiments were conducted with different lots of chicks, the greatest difference in the average growth rates of groups fed identical diets was 0.7%, this figure has been used as the limit of significance.

RESULTS

Corn gluten meal

In table 1, the growth results with the various supplements to the corn gluten meal diet are given. The growth rate was raised from 1.9% (diet 1) to 5.6% (diet 2) by the addition of arginine, cystine, glycine, lysine and tryptophane. The effect on growth of each of these amino acids (together with methionine, threonine and valine) was studied with the corn gluten meal ration. These particular amino acids were studied either because the analytical data indicated a possible deficiency, or because the analytical or requirement data were in doubt.

Arginine. According to Block and Bolling ('45), corn gluten meal protein contains 3.2% arginine. When the total protein content of the diet is 20%, the arginine present in the diet is 0.64%, which is less than the requirement of 0.9% (Almquist, '45). As was expected from these data, omission of arginine from the supplement decreased growth (from 5.6%, diet 2, to 4.3%, diet 3).

Lysine. According to the analytical data, about 40% of the required amount of lysine is furnished by corn gluten meal protein. The necessity of supplementation with lysine is clearly shown by the comparison of diet 2 with diet 4, where, when lysine was omitted, the growth was no better than that found with the basal diet (1).

Tryptophane. From chemical analyses (Kratzer, '44), a tryptophane deficiency in corn gluten meal protein was ex-

TABLE 1

Effects of various supplements to the corn gluten meal diet on the rate and efficiency of gain. The concentrate provided 20% protein (N × 6.25) in the diet. The numbers in parentheses are cross-references to other diet numbers. These allow direct comparison of the effects of supplementation by the amino acid at the head of the column.

DIET NUMBER	SUPPLEMENTS TO THE BASAL DIET [1]								NO. OF GROUPS	AVE. % GAIN PER DAY	GAIN/FEED
	l(+) Arginine	l(+) Lysine	l(−) Tryptophane [2]	l(−) Cystine	Glycine	dl-Methionine	dl-Threonine	dl-Valine			
1	..	...	..	..	..	..	..	..	2	1.9	0.18
2	0.3 (3)	0.55 (4)	0.2 (5)	0.3 (6)	0.8 (10)	.. (12)	.. (17)	.. (14, 15)	4	5.6	0.42
3	.. (2)	0.55	0.2	0.3	0.8	..	..	..	1	4.3	0.33
4	0.3	... (2)	0.2	0.3	0.8	..	..	..	1	1.5	0.16
5	0.3	0.55	.. (2)	0.3	0.8	..	..	..	1	1.7	0.16
6	0.3	0.55	0.2	.. (2)	0.8	..	..	..	2	5.7	0.42
7	0.3	0.55	0.2	0.3 (8)	0.8 (13)	0.2 (14, 15)	..	0.5 (12)	1	5.6	0.52
8	0.3	0.55	0.2	.. (7, 9)	0.8	0.2	..	0.5	1	5.5	0.51
9	0.3	0.55	0.2	0.3 (8)	0.8 (13)	0.1 (14, 15)	..	0.5 (12)	1	5.8	0.45
10	0.3	0.55	0.2	0.3	.. (2)	..	..	..	2	5.5	0.41
11	0.3	0.55	0.2	0.3	.. (16)	..	0.5	0.3	1	5.9	0.44
12	0.3	0.55	0.2	0.3	0.8	0.2 (2)	..	.. (7, 9)	1	5.7	0.51
13	0.3	0.55	0.2	0.3	.. (7, 9)	0.2	..	0.5	1	5.9	0.45
14	0.3	0.55	0.2	0.3	0.8	.. (7, 9)	.. (16)	0.5 (2)	1	5.9	0.51
15	0.3	0.55	0.2	0.3	0.8	.. (7, 9)	.. (16)	0.5 (2)	1	6.2	0.47
16	0.3	0.55	0.2	0.3	0.8 (11)	..	0.5 (14, 15)	0.5 (17)	1	5.8	0.44
17	0.3	0.55	0.2	0.3	0.8	..	0.5 (2)	.. (16)	1	5.8	0.44

[1] Expressed as per cent of the diet.

[2] In some experiments, 0.4% dl-tryptophane replaced 0.2% l(-)tryptophane.

pected, and this was confirmed by growth studies. When tryptophane was omitted from the supplements of diet 2, growth was decreased from 5.6% to 1.7% (diet 5).

Cystine. The large variation in the reported cystine content of corn proteins made it desirable to test the effect of this amino acid in the absence, and presence, of methionine. Omission of cystine had no significant effect (compare diet 2 with diet 6, and diets 7 and 9 with diet 8).

Glycine. Like that of cystine, the glycine content of corn gluten meal is not accurately known, and therefore trials were made using glycine supplements. Comparisons of diets 2 and 10, 11 and 16, 7 and 13, and 9 and 13 show no significant effect of the glycine supplement.

Methionine. Corn gluten meal protein was found to contain 2.3% methionine (Grau and Almquist, '45a) when analyzed by the colorimetric method of McCarthy and Sullivan ('42). Since this is probably barely enough to satisfy the methionine requirement (Grau and Almquist, '43), its effect as a supplement was tried. Comparisons of diet 2 with diet 12, diets 7 and 9 with diet 12, and diets 7 and 9 with diets 14 and 15 show that the corn gluten meal diets are not deficient in methionine.

Threonine. The threonine requirement has been in such doubt that some trials were made to determine its possible effect on growth with the corn gluten meal diet. Neither the addition of threonine to diet 2 (as in diet 17) nor to diet 15 (as in 16) gave any increase in growth.

Valine. No effect from valine supplementation was found (diet 2 and diets 14 and 15; diet 12 and diets 7 and 9).

The best rate of growth with the corn gluten meal diets was about 6%, while groups fed supplemented peanut or cottonseed meals showed rates of about 7%, which is the rate obtained with commercial-type rations. It is, of course, possible that some one amino acid is not present in sufficient amount in corn gluten meal; an alternative explanation is that those amino acids which are present occur in proportions which are too different from the optimum to allow best growth.

Cottonseed meal

The amino acid analyses of cottonseed meal point to deficiencies of lysine, methionine, and (possibly) tryptophane. In addition to these amino acids, the effects of supplementing with threonine were studied.

When lysine alone was added to the basal diet, growth was increased from 4.4% (table 2, diet 18) to 5.7% (diets 19 and 20). A similar addition of methionine (diet 21) had no such effect, but the addition of both lysine and methionine (diet 22) allowed a growth of 6.9%.

Threonine supplementation was ineffective (compare diet 24 with diet 23), as was that of tryptophane (diets 25 and 22).

Peanut meal

According to amino acid analyses, the principal deficiencies of peanut meal are those of lysine and methionine, and possibly threonine and tryptophane. Various combinations of these amino acids were added to the basal diet, with the results shown in table 2 (diets 26 through 35).

The basal diet gave poor growth (3.3%, diet 26) which was not increased by the addition of lysine alone (3.1%, diet 27). Methionine, on the other hand, had a definite effect on growth when fed alone (5.8%, diet 28). When the basal diet was supplemented with all four of the amino acids mentioned above, the growth rate was 6.9% (diet 29). When lysine was omitted, growth was decreased only to 5.5% (diet 31) but when methionine was omitted, the resulting growth was 2.7% (diet 32).

The possible supplementary effect of threonine was investigated with diets 29 and 33, 34 and 35, and 34 and 33. Although the difference in growth rate between diets 34 and 33 exceeded 0.7%, the differences within the other two pairs were less than this value, so that the effect of threonine cannot be considered significant.

Omission of tryptophane had no effect, whether threonine was present (diets 29 and 34) or absent (diets 33 and 35).

TABLE 2

Effects of various supplements to the cottonseed meal and peanut meal diets on the rate and efficiency of gain. The numbers in parentheses are references to diet numbers.

PROTEIN CONCENTRATE	DIET NO.	SUPPLEMENTS TO THE BASAL DIET [1]				NO. OF GROUPS	AVE. % GAIN PER DAY	GAIN/FEED
		l(+) Lysine	dl-Methionine	dl-Threonine	l(−) Tryptophane [2]			
Cottonseed meal	18	... (19)	.. (21)	..	..	1	4.4	0.30
	19	0.46 (18)	..	..	..	2	5.7	0.38
	20	0.37 (18)	..	..	..	1	5.7	0.41
	21	...	0.2 (18)	..	..	1	4.3	0.27
	22	0.46 (18, 19)	0.2 (18)	..	.. (25)	2	6.9	0.45
	23	0.37	0.2	.. (24)	..	2	6.3	0.43
	24	0.37	0.2	0.3 (23)	..	1	6.2	0.43
	25	0.46	0.4	..	0.1 (22)	1	6.6	0.44
Peanut meal	26	... (27)	.. (31)	..	..	1	3.3	0.28
	27	0.46 (26)	..	..	..	1	3.1	0.26
	28	...	0.2 (26)	..	..	2	5.8	0.38
	29	0.46 (31)	0.4 (32)	0.5 (33)	0.1 (34)	1	6.9	0.52
	30	0.46	0.1	0.5	..	1	7.0	0.43
	31	... (29)	0.4	0.5	0.1	1	5.5	0.40
	32	0.46	.. (29)	0.5	0.1	1	2.7	0.25
	33	0.46	0.4	.. (29, 34)	0.1 (35)	3	6.4	0.48
	34	0.46	0.4	0.5 (33)	.. (29)	2	7.5	0.57
	35	0.46	0.2	..	.. (33)	2	7.0	0.49

[1] Expressed as per cent of the diet.

[2] In some experiments, 0.2% dl-tryptophane replaced 0.1% l(−)tryptophane.

SUMMARY

Diets containing corn gluten meal, cottonseed meal or peanut meal were fed to young chicks so that all the 20% crude protein of each diet was provided by one of these concentrates. Additions of various amino acids were made, and the effects on growth and efficiency of gain were noted.

Corn gluten meal required the addition of arginine, lysine and tryptophane in order to increase the rate of growth from 2% to slightly less than 6% per day. Cystine, glycine, methionine, threonine and valine were already present in adequate amounts.

Supplementation of cottonseed meal with both methionine and lysine increased the growth rate from 4% to 7%. The methionine deficiency was found to be less marked than that of lysine, a result which was expected from amino acid analyses.

Peanut meal is lacking primarily in methionine, but is also slightly deficient in lysine.

ACKNOWLEDGMENTS

This study was aided by a grant from Swift and Co. Valuable technical assistance was provided by Miss I. H. Klaus.

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THE NUTRITIVE VALUE OF THE PROTEIN OF VARIETIES OF LEGUMES AND THE EFFECT OF METHIONINE SUPPLEMENTATION [1, 2]

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(Received for publication May 4, 1946)

Although many studies have been made of the nutritive value of the protein of legumes, little attention has been given to the question of whether the nutritive value differs with respect to variety. The proteins of none of the common legumes, peas, lima beans, and snap beans, have been reported to promote growth in the white rat as well as that of soybeans, and it was of interest to find out whether some varieties of the commonly used legumes contain protein of higher nutritive value than others and whether any equal that of the soybean. Furthermore, the observation of Almquist and associates ('42) on the supplementing effect of methionine for soybean protein, those of Woods, Beeson and Bolin ('43) on the supplementary effect of this amino acid for Alaska field peas, and the results of preliminary studies in our own laboratory led to a study of the effect of methionine supplementation and the determination of the methionine content of the legumes studied.

[1] Journal Series paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Agricultural Biochemistry.

[2] Presented before the Division of Agricultural and Food Chemistry, American Chemical Society, Atlantic City, New Jersey, April 8–12, 1946.

EXPERIMENTAL

In preliminary studies it had been observed that the use of the common legumes, lima beans, peas or snap beans, as the sole source of protein, at the 8 to 10% protein level, in a purified diet, either failed to promote growth or caused only slight gains. Whenever the diet was supplemented with methionine, however, there was an immediate increase in growth rate; and when the amino acid was removed from the diet there was an immediate loss of weight, indicating that storage of this nutrient had not taken place. None of the other essential amino acids, fed singly, caused a significant response. Woods and associates ('43) have reported marked improvement of growth rate when the protein of Alaska peas was supplemented with methionine. In the present experiment it was further noted that the same conclusion as to growth promoting properties was reached after a 10-day experimental period as after 20 or 28 days. Thus for Kentucky Wonder lima beans the average daily gain during the first 10 days of feeding was 0.2 gm, for the second 10 days 0.4 gm and for the final 8 days of a 28-day period, 0.1 gm. The results of the whole 28-day period, or of any one of the three periods, showed that the nutritive value of the protein of this variety of legume was very poor. Consequently, for the purpose of determining whether the proteins of the varieties of legumes were of good or poor quality, 10-day feeding periods were used. To determine the growth promoting properties of the protein of the varieties of legumes, and the effect of methionine supplementation, on a relative basis, white rats were fed a basal diet for a 10-day period, which was followed by a second 10-day period during which 0.1% methionine was incorporated in the diet, and by a third 10-day period during which 0.6% methionine was supplied. Finally, for a fourth 10-day period, the animals were returned to the basal diet. Three male and three female rats, 22 to 26 days of age and weighing 55 to 65 gm comprised each group.

Legume seeds of known variety were obtained from seed supply houses,[3] washed, soaked overnight, cooked in the soaking water as if for human consumption and dried at 50°–60°C. Green snap beans were likewise cooked as for human consumption and prepared for feeding as were the dried seeds. The dried whole beef, reported for the purpose of comparison, was prepared by drying thin slices of raw, frozen, ground lean beef at 55°–60°C. All of the dried products were ball milled to a fine powder before feeding.

Each diet contained salt mixture U.S.P., 4%; sodium chloride, 1%; cane sugar, 5%; and cod liver oil, 1%. Three to 4% corn oil was added, depending upon the fat content of the dried legume, so that the total fat content of the diet was 5 to 6%. The dried ground legume was incorporated in the diet to supply 10% protein, and the total of ingredients was brought to 95% by the addition of starch. When used as a basal, unsupplemented diet, 5% cane sugar was added, and to supplement the diet with methionine, the calculated quantity was incorporated in the 5% of cane sugar. For each 100 gm of diet the following quantities of water soluble vitamins, and other organic substances, were added in an alcoholic solution and dried on the diet: thiamine, 0.5 mg; riboflavin, 1.0 mg; pyridoxine, 0.5 mg; calcium pantothenate, 2.5 mg; nicotinic acid, 2.0 mg; inositol, 0.25 mg; p-aminobenzoic acid, 5.0 mg; choline, 20 mg and biotin 2 μg.

Methionine was determined by the Hess and Sullivan ('43) modification of the McCarthy and Sullivan ('41) method. Hydrolysis of the protein was accomplished by heating with 20% HCl in a sealed tube at 125°C. for 3 hours, as suggested by Hess and Sullivan ('45).

RESULTS AND DISCUSSION

In table 1 average daily gains, average daily food consumption and gains per gram of protein consumed are shown

[3] For the supply of seeds, we are indebted to the Associated Seed Growers, Inc., New Haven, Conn., Alexander Forbes and Company, Newark, N. J., and F. H. Woodruff and Sons, Inc., Milford, Conn.

for four varieties of lima beans (Phaseolus lunatus), five varieties of snap beans (Phaseolus vulgaris) and nine varieties of peas (Pisum sativum), when fed with and without methionine. In addition data are shown for one variety of soybeans (Soja Max.), for chick peas (Cicer arientinum) and, for purposes of comparison, for dried whole beef. The varieties of lima beans, peas and snap beans have been arranged in descending order of the gains per gram of protein consumed when the diet was supplemented with 0.1% methionine.

None of the varieties of lima beans, snap beans or peas showed more than slight growth during the first period on the basal diet and therefore the differences in the nutritive value of the proteins of these varieties were not significant. The largest average daily gains, 0.4 gm for King of the Garden lima beans and for Laxtonian peas, were significantly less than the responses of 1.6 gm and 1.2 gm for soybeans and chick peas, respectively. These marked differences in response were also apparent in the gains per gram of protein consumed.

When 0.1% methionine was added to the basal diets, the response was immediate for all of the varieties of lima beans, snap beans and peas, as well as for the soybeans and chick peas. During the first 24 hours of methionine feeding the gains ranged from 1 gm for some of the varieties of snap beans and peas to 7 gm in the case of the King of the Garden lima beans. Whereas no significant differences in growth response occurred when the basal ration was fed, poor growth being observed for all of the varieties of lima beans, snap beans and peas, supplementation with methionine accentuated the differences in the nutritive value of the proteins so that the gains per gram of protein consumed ranged from 0.9 gm for the Stringless Green Pod snap beans to 3.0 gm for the King of the Garden variety of lima beans. These results lead to the conclusion that there are differences in the nutritive value of the protein of these varieties when they are supplemented with methionine. The gain of 3.0 gm per gram of protein consumed, shown by the King of the Garden lima

TABLE 1

Varieties of cooked dried legumes fed at the 10% protein level. Six rats in each group (3 ♂'s and 3 ♀'s). Average daily growth response, average daily food consumption and gain per gram of protein consumed for 10 days

TABLE 1

Varieties of cooked dried legumes fed at the 10% protein level. Six rats in each group (3 ♂'s and 3 ♀'s). Average daily growth response, average daily food consumption and gain per gram of protein consumed for 10-day period.

SPECIES AND VARIETY	PROTEIN CONTENT	BASAL DIET			BASAL DIET + 0.1% METHIONINE			BASAL DIET + 0.6% METHIONINE			BASAL DIET	
		Gain	Food consumption	Gain per gm protein consumed	Gain	Food consumption	Gain per gm protein consumed	Gain	Food consumption	Gain per gm protein consumed	Change in body wt.	Food consumption
	%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Lima beans (Phaseolus lunatus)												
King of the Garden	20.00	+ 0.4	6.1	0.7	3.0	9.8	3.0	3.7	12.0	3.1	— 0.7	11.5
Fordhook	20.20	+ 0.3	4.8	0.6	1.3	6.5	2.0	2.1	8.2	2.6	— 1.2	6.4
Baby Fordhook	19.44	+ 0.1	5.3	0.2	1.4	10.6	1.3	2.1	11.1	1.9	+ 0.2	8.8
Challenger Pole	19.50	— 0.4	5.4	..	1.3	10.6	1.2	2.2	12.9	1.7	— 0.5	7.4
Snap beans (Phaseolus vulgaris)												
Sure Crop Wax	25.38	— 0.1	5.5	..	1.6	8.0	2.0	1.6	8.4	1.9	— 0.7	6.4
Pencil Pod	25.63	— 0.3	5.6	..	1.5	8.2	1.8	1.6	8.4	1.9	— 0.2	7.7
Bountiful	19.19	+ 0.1	5.8	0.2	1.3	7.6	1.7	1.8	8.0	2.3	+ 0.5	6.6
Kentucky Wonder	24.80	— 0.5	4.1	..	0.7	6.9	1.0	2.2	7.6	2.9	— 0.2	6.7
Stringless Green Pod	22.75	— 0.4	5.2	..	0.5	5.5	0.9	1.9	6.8	2.8	0.0	6.7
Peas (Pisum sativum)												
Alderman	26.56	+ 0.2	5.3	0.4	2.0	7.7	2.6	1.7	8.5	2.0	0.0	7.4
Laxton's Progress	29.90	+ 0.3	6.6	0.5	2.3	9.8	2.3	2.8	12.7	2.2	— 1.5	12.4
Alaska 2 B	26.13	— 0.5	5.3	..	2.0	8.6	2.3	1.4	8.6	1.6	— 0.2	7.9
World's Record	29.00	+ 0.2	5.0	0.4	1.7	7.6	2.2	1.8	7.3	2.5	— 0.7	5.4
Laxtonian	27.12	+ 0.4	6.1	0.7	1.9	8.9	2.1	2.0	11.6	1.7	— 0.2	9.1
Little Marvel	25.60	+ 0.2	5.0	0.4	1.4	7.2	1.9	1.8	7.4	2.4	— 0.8	8.3
Thomas Laxton	29.00	+ 0.3	6.8	0.4	1.6	8.9	1.8	2.1	10.9	1.9	— 0.5	10.6
Mammoth Luscious	26.30	0.0	6.3	..	1.6	9.1	1.8	2.0	12.1	1.7	— 0.7	11.5
Teton	27.90	— 0.2	5.8	..	0.8	6.8	1.2	1.8	8.8	2.0	— 0.9	7.9
Soybeans (Soja Max.)												
Bansei	41.50	1.6	7.5	2.1	3.3	11.6	2.8	3.4	13.0	2.6	+ 1.2	11.1
Chick peas (Cicer arientinum)	21.64	1.2	6.6	1.8	2.5	9.4	2.7	2.8	10.0	2.8	— 2.4	9.8
						(No methionine)						
Dried whole beef	82.81	3.1	..	..	3.7	11.0 [1]	3.0 [1]					

[1] For the 20-day period.

beans, was practically the same as that produced when dried whole beef (table 1) was fed. Therefore, the protein of this variety of lima beans, when supplemented with methionine, and that of dried whole beef, have essentially the same nutritive value. There were no essential differences among the average daily gains of the three other varieties of lima beans, all of them being markedly lower than that of the King of the Garden variety. For each of two varieties, Baby Fordhook and Challenger Pole, the gains per gram of protein consumed were essentially the same, but a somewhat higher efficiency of gain was recorded for the Fordhook variety.

When 0.1% methionine was added to the basal diets containing protein supplied by snap beans, the average daily gains varied from 0.5 gm to 1.6 gm, and the gains per gram of protein consumed, 0.9 gm to 2.0 gm, placed the varieties in the same order as did the daily gains. Both of these ranges of values are essentially the same as those of the varieties of lima beans and peas which showed the poorest responses.

The incorporation of 0.1% methionine in the basal pea diets caused five varieties to show average daily gains, from 1.7 gm to 2.3 gm, and gains per gram of protein consumed, from 2.1 gm to 2.6 gm, which were higher than those of any of the varieties of snap beans and lima beans, except the King of the Garden variety of the latter species. The remaining four varieties of peas showed responses of the same order as those of the snap beans and the poorer varieties of lima beans.

Since only one variety, the King of the Garden, had shown a gain of the same order as that observed for meat, a protein of high quality, and since the animals were still in an actively growing stage, it was desirable to determine whether an increase in methionine percentage would stimulate further growth. Consequently, the level was raised to 0.6%, that proposed by Rose ('37) as meeting the minimum requirements for this amino acid, at the close of the 10-day period during which 0.1% methionine was fed.

Each of the varieties of lima beans showed a marked increase in rate of gain, the average daily gain for three of

them being 2.1 gm to 2.2 gm, as well as in gain per gram of protein consumed. For the King of the Garden variety, the average daily gain became 3.7 gm, the same as that recorded for meat protein in the second 10-day period. The gain per gram of protein consumed remained essentially the same as that observed in the 0.1% methionine period, however, because of the increased food consumption.

Higher average daily gains resulted from the varieties of snap beans, except for the Sure Crop Wax variety, for which the rate of gain remained the same. Two varieties, Kentucky Wonder and Stringless Green Pod, showed increases in rates of gain of 1.5 gm and 1.4 gm, respectively. For these two varieties the gain per gram of protein consumed was almost as great as that calculated for the King of the Garden variety, but the body weights of the animals were much less than those receiving the King of the Garden diet.

In general, the responses when the varieties of peas were fed with the higher level of methionine were inferior to those of lima beans and snap beans. Actually, for two varieties, Alderman and Alaska, there was a lowering of the average daily rate of gain, accompanied by a drop in the gain per gram of protein consumed. An explanation for the lower responses is not apparent. The World's Record and Laxtonian varieties showed virtually no change in rate of gain, but a marked increase in consumption of the Laxtonian diet lowered the gain per gram of protein consumed. For each of the remaining five varieties there was an increase in the rate of gain and for the Teton variety a significant rise in the gain per gram of protein consumed.

When methionine was removed from the diet, and the animals were provided with only the basal ration for a 10-day period, for all of the varieties of lima beans, snap beans and peas immediate cessation of gain and loss of weight occurred, except for one variety of lima beans, the Baby Fordhook, which showed a slight gain. This behavior leads to the conclusion that there is no storage of methionine such as that observed with the vitamins. It is of interest that in many cases

the food consumption remained essentially the same as during the period when 0.6% methionine was fed.

The good nutritive quality of soybean protein is well known, and one variety (Bansei) of this species was included for comparison. During the first basal period (table 1), the average daily gain, 1.6 gm, and the gain per gram of protein consumed, 2.1 gm, were markedly superior to that of any of the varieties of lima beans, snap beans and peas. When 0.1% and 0.6% methionine were fed, the average daily gains exceeded those of all of the varieties except that of the King of the Garden variety. In this instance the responses were of the same order. The higher food consumption by the animals of the soybean group resulted in gains per gram of protein slightly lower than those calculated for the lima bean variety. It is also of interest that the responses when the soybeans were supplemented with methionine are of the same order as those reported in table 1 for dried whole beef. During the basal period the animals on the soybean diet continued to grow in contrast with those on all of the other legume diets, for which losses or, at the most, slight gains, were noted. Thus of the twenty legumes tested, only the soybeans provided sufficient methionine to support growth in the final basal period.

The relatively high nutritive value of chick peas (Cicer arientinum), also called "garbanza" (Spanish-speaking countries), "gram" (India) and "ceci" (Italy), observed in earlier studies led to a test of the effect of methionine supplementation of this legume. As noted in table 1, during the first basal period the gain per gram of protein consumed, though not so great as that shown by soybeans, was far higher than that of the best of the responses in the varieties test. During the two periods of methionine feeding the daily gain and the gain per gram of protein consumed were less than the values for soybeans and the King of the Garden variety but better than any of the other varieties except Laxton's Progress peas which showed daily gains of the same order as those of the chick peas. The gains per gram of protein

consumed in the case of the Laxton's Progress variety, however, were somewhat less. During the final basal period the chick peas differed from the soybeans in that there was a loss in weight, probably due to insufficient available methionine for maintenance and growth of animals that were considerably heavier than during the first basal period. The results with chick peas as the sole source of protein in the dietary confirm an earlier observation reported by Mitchell ('28).

TABLE 2

Varieties of green snap beans fed at the 10% protein level. Four rats in each group (2 ♂ and 2 ♀). Average daily growth response, average daily food consumption and gain per gm of protein consumed for 10-day periods.

CATEGORY OF INTEREST	SNAP BEANS (PHASEOLUS VULGARIS) VARIETY		
	Tender Green	Giant Stringless	Black Valentine
Basal diet			
Protein content in %	22.19	21.63	20.06
Change in body weight in gm	— 0.5	— 0.3	— 0.3
Food consumption in gm	4.8	4.6	5.5
Basal diet + 0.6% methionine			
Change in body weight in gm	0.0	+ 0.5	+ 0.5
Food consumption in gm	5.5	6.8	7.0
Gain per gm protein consumed in gm	..	0.7	0.7

The protein contents of the cooked dried products, shown in tables 1 and 2, do not bear a relation to the nutritive value of the protein either unsupplemented or when supplemented with methionine. Thus, for example, the protein content of the King of the Garden variety of lima beans is 20.00% and the gain per gram of protein consumed, when 0.1% methionine was fed, was 3.0 gm. In contrast, for the Teton variety of peas, whose protein content is 27.90%, the gain per gram of protein consumed was 1.2 gm.

During the tests of the varieties of legumes in the form of dried seeds, three varieties of green snap beans became

available from the experimental plots of the Department of Vegetable Gardening.[4] Accordingly, they were tested in the same manner as the varieties of dried legumes. Although the protein content (table 2) of the cooked, dried, green snap beans was within the range of values obtained with varieties in the dried form (table 1), none of the three green varieties promoted growth when fed as the sole source of protein at the 10% protein level. As sufficient quantities were not available for a complete test, only the supplementation with 0.6% methionine was tried. As noted in table 2, body weight was maintained on the Tender Green variety and very slight average daily gains, and gains per gram of protein consumed, resulted with the other two varieties, the gains being definitely less than any of those observed with the dried varieties. Further study will be necessary to ascertain the cause of this difference, which may be due, for example, to the dilution of seed protein by pod protein or to the possibility that a more complete pattern of amino acids develops as the seed matures.

Following the animal experiments just described, the methionine content was determined on all of the legumes studied, except the Fordhook variety of lima beans and the Little Marvel variety of peas. The results are presented in table 3, along with the gains per gram of protein consumed during the first basal period and when the supplement was 0.1% methionine, in order of the decreasing magnitude of the methionine contents of the diets.

The growth response when the basal diets were fed or when they were supplemented with methionine did not bear a direct relationship to the methionine contents of the diets. Thus, the basal diet made from Laxtonian peas contained 0.30% methionine and the gain per gram of protein consumed was 0.7 gm whereas that made from soybeans contained 0.16% and from chick peas 0.14%, yet the gains per gram of protein consumed were significantly higher, being 2.1 gm and 1.8 gm.

[4] Prof. L. G. Schermerhorn of the Department of Vegetable Crops, Rutgers University, very kindly supplied these varieties.

respectively. These results indicate that the methionine of soybeans and chick peas is more readily available to the rat than that in Laxtonian peas. The failure to obtain more than slight growth on the Laxtonian pea diet is not due to an inadequacy of other essential amino acids because supplementation with 0.1% of crystalline methionine, one-third of the quantity already present in the Laxtonian pea diet, resulted

TABLE 3 [1]

Methionine content of legumes and of diets, and growth responses.

LEGUME	NITROGEN CONTENT, OVEN-DRIED BASIS	METHIONINE CONTENT, OVEN-DRIED BASIS	METHIONINE IN BASAL DIET	GAIN PER GRAM OF PROTEIN CONSUMED	
				Basal diet	Basal diet + 0.1% dl methionine
	%	%	%	gm	
Laxtonian peas	4.60	0.85	0.30	0.7	2.1
Stringless Green Pod snap beans	3.89	0.57	0.23	loss	0.9
Bountiful snap beans	3.24	0.45	0.22	0.2	1.7
Baby Fordhook lima beans	3.38	0.41	0.20	0.2	1.3
Kentucky Wonder snap beans	4.24	0.46	0.17	loss	1.0
King of the Garden lima beans	3.32	0.34	0.17	0.7	3.0
Soybeans	7.73	0.75	0.16	2.1	2.8
Challenger Pole lima beans	3.36	0.34	0.16	loss	1.2
Sure Crop Wax snap beans	4.25	0.42	0.16	loss	2.0
Chick peas	3.70	0.32	0.14	1.8	2.7
Pencil Pod snap beans	4.42	0.35	0.13	loss	1.8
Thomas Laxton peas	4.88	0.38	0.12	0.4	1.8
Teton peas	4.74	0.35	0.12	loss	1.2
Laxton's Progress peas	5.05	0.37	0.12	0.5	2.3
Alaska 2 B peas	4.52	0.30	0.11	loss	2.3
Alderman peas	4.53	0.29	0.10	0.4	2.6
Mammoth Luscious peas	4.53	0.29	0.10	loss	1.8

in a gain of 2.1 gm per gram of protein consumed. The same conclusion may be drawn with regard to all of the varieties of lima beans, snap beans, and peas listed in table 3, since even in the case of those which provided the lowest percentage of methionine in the diet, the Alderman and Mammoth Luscious varieties of peas, the quantity was 0.1%, an amount which fed as a crystalline supplement did promote growth.

These observations emphasize the danger that may be encountered in using the results of the amino acid analysis of proteins for the compounding of rations and the desirability of testing the results of chemical and microbiological analyses by animal experiments.

It is of further interest that the methionine provided by the King of the Garden variety of lima beans, along with the supplementary level of 0.1%, made a total of 0.25% in the diet. The gain recorded for this variety was of a high order, 3.0 gm per gram of protein consumed, yet the total quantity of methionine in the diet, some of which was not available, was less than half of the 0.6% level of methionine given by Rose ('37) as the quantity of the crystalline acid which must be fed along with the recommended amounts of the other essential acids and liberal quantities of the non-essential amino acids, to induce optimum growth.

SUMMARY

1. Since none of four varieties of lima beans (Phaseolus lunatus), five varieties of snap beans (Phaseolus vulgaris) and nine varieties of edible peas (Pisum sativum), when fed as the sole source of a 10% protein level, promoted more than slight growth in the white rat, it is concluded that there are no significant differences in the nutritive value of their unsupplemented proteins. The responses were markedly less than those of soybeans (Soja Max.) and chick peas (Cicer arientinum) fed under the same conditions.

2. The addition of 0.1% methionine to the basal diet caused an immediate growth response and increase in the gain per gram of protein consumed for all of the varieties of lima beans, snap beans and peas, as well as for soybeans and chick peas, and with this supplementation differences in the nutritive value of the protein of these varieties become apparent.

3. When the level of methionine was raised to 0.6%, there was a further growth response and gain per gram of protein consumed except in the case of three varieties of peas for which losses occurred.

4. Of the eighteen varieties of lima beans, snap beans and peas tested, only the protein of the King of the Garden variety of lima beans, when supplemented with methionine, promoted average daily gains and gains per gram of protein consumed of the same order as those obtained with soybeans supplemented with methionine and with dried whole beef.

5. The nutritive value of the protein of chick peas (also called "garbanza," "gram" and "ceci") is almost as high as that of the soybeans.

6. When three varieties of snap beans, picked green, were fed in the cooked and dried form as the sole source of a 10% protein level, they failed to promote growth and the addition of 0.6% methionine resulted in only very slight gains for two of the varieties.

7. The methionine content of the legumes, on the oven-dried basis, ranging from 0.29 to 0.85%, did not have a direct relationship to nutritive value of the proteins.

8. The methionine of soybeans and chick peas is more readily available to the rat than that present in the varieties of lima beans, snap beans and peas studied.

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INFLUENCE OF A SINGLE DOSE OF ALPHA TOCOPHEROL ADMINISTERED TO VITAMIN E DEFICIENT RATS ON THE FIFTEENTH DAY UPON SUBSEQUENT GROWTH [1]

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FIVE FIGURES

(Received for publication May 21, 1946)

In cooperation with Pappenheimer and his associates (Kaunitz et al., '44; Pappenheimer et al., '44), it was shown that the administration of a single dose of alpha tocopherol to rats on a tocopherol deficient diet delays the onset and decreases the degree of testicular atrophy. The effect of 1 mg alpha tocopherol was most pronounced when given on the fifteenth day of life, less definite on the twenty-eighth day and inapparent when given on the sixth day of life. Similarly, the oxygen consumption of vitamin E deficient rats was influenced over a period of several months when 1 mg alpha tocopherol was fed on the fifteenth day (Kaunitz and Pappenheimer, '43).

The influence of tocopherol on growth has been studied before (Evans, '28; Olcott and Mattill, '36). Rats on a tocopherol low diet show growth deficit several months after birth. It seemed worthwhile to ascertain whether this effect could also be influenced by a single dose of tocopherol given during the lactation period.

[1] Aided by a grant from the John and Mary R. Markle Foundation.

METHODS

The data presented in this study were obtained from a colony of albino rats maintained on two different diets. One group and its ancestry for six generations had been kept on the "Evans-Burr" diet, consisting of: casein, 32 parts; cornstarch, 40 parts; yeast, baker's dried, 10 parts; lard, commercial, 22 parts; salts (Hawk-Oser), 4 parts and cod liver oil, 2 parts.

The second diet (table 1) used was of "semi-synthetic" character and was developed in cooperation with Dr. Charles Slanetz.[2]

TABLE 1

Composition of tocopherol-low diet used.

BASAL MIXTURE		SUPPLEMENTS OF BASAL MIXTURE	
	%		*mg/kg*
Lard	10	Thiamine chloride	2
Casein [1]	30	Riboflavin	4
Cerelose	54	Pyridoxine	4
Celluration	2	Nicotinic acid	100
Salt mixture [2]	4	Choline	1000
		Vitamin K	4
		Para-amino-benzoic acid	300
		Ca pantothenate and Percomorphum oil (ml/kg)	10 0.2

[1] Borden's crude no. 453.
[2] Hawk-Oser.

The tocopherol content of this diet was repeatedly examined with the Emmerie-Engel reagent (Emmerie and Engel, '38; Devlin and Mattill, '42), according to the internal standard method of Kaunitz and Beaver (Kaunitz and Beaver, '44). It contained definitely less than 0.3 mg, and probably less than 0.1 mg tocopherol per 100 gm of diet.

When mothers reared on the "Evans-Burr" diet had a litter, they were immediately placed on the semi-synthetic diet. Eighteen virginal females, reared on this diet, were successfully mated; no "first litter fertility" was observed.

[2] We are indebted to the Abbott Laboratories for the choline and the synthetic vitamins.

On the fifteenth day of life some of the young were given 1 drop of sesame oil containing the desired amounts of synthetic dl alpha tocopherol,[3] the untreated litter mates serving as controls.

Further studies were made on the testes. First the right and, after a further period, the left one was removed under brief ether narcosis. In animals under 60 days of age, no adverse effect on the growth was observed 1 week after semi-castration. In older males, the period of recovery lasted sometimes 2 to 3 weeks. For the studies of the motility of the sperm, epididymis content was examined in saline solution.

RESULTS

I. Effect on growth

Zucker and Zucker ('42) observed that the optimal weight curve of healthy rats maintained on a complete diet is a straight line when the weight is plotted as the abscissa on a logarithmic scale and the ordinate represents the reciprocal value of the age. The slope of the line is constant for rats of the same sex (fig. 1). Deviations from the line of optimal growth represent a weight deficit which can be expressed in per cent of the optimal weight.

Figure 1 demonstrates that the mean weights of male rats on the semi-synthetic diet without additional doses of tocopherol begin to deviate from the optimal growth at the age of roughly 7 weeks; at the age of about 21 weeks, their weight deficit is about 20% and thereafter they actually lose weight. Male rats that had received a single dose of tocopherol of 1, 5 or 27 mg on the fifteenth day show a smaller weight deficit and their weight continues to increase after the twenty-first week.

The individual variations of the results and their statistical evaluation are given in figure 2. The difference between the two groups is statistically highly significant; no differences of

[3] Hoffmann-La Roche very kindly supplied us with synthetic dl alpha tocopherol.

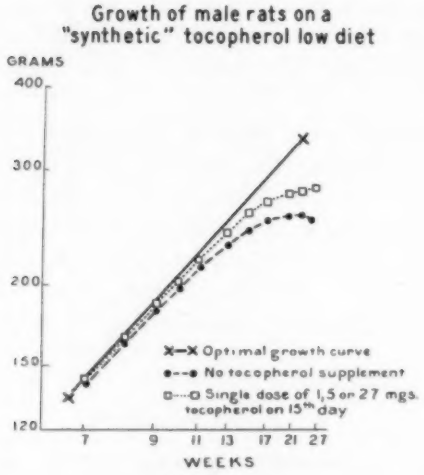

Figure 1

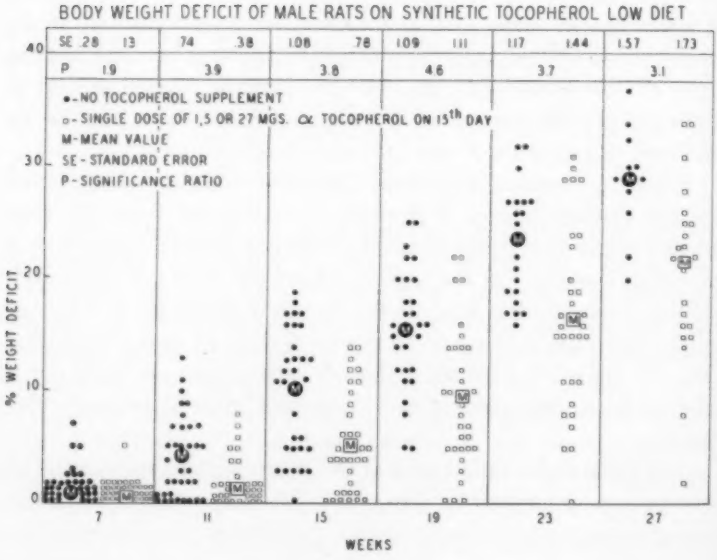

Figure 2

the mean values were noted among those groups that had received graded doses of tocopherol.

In nine male rats of the same colony raised on the semi-synthetic diet augmented by 3 mg synthetic dl alpha tocopherol per 100 gm of diet, the mean weight deficit was similar to that of the group that had received a single dose of alpha tocopherol on the fifteenth day.

Figure 3 demonstrates the weight deficits of male rats raised on the "Evans-Burr" diet.[4] Again it was noted that the mean weight deficit of the group that received a single dose of 1, 5 or 27 mg tocopherol on the fifteenth day was less than that of the "unprotected" males. The statistical significance of the difference of the mean values is less pronounced than in the groups on semi-synthetic diet (fig. 2), probably because fewer rats on the "Evans-Burr" diet were used.

When synthetic dl alpha tocopherol of from 2.5 to 10 mg per 100 gm was incorporated in the "Evans-Burr" diet, the mean weight deficit observed in a group of nineteen males was 7% after 21 weeks, as compared to 12% in the group with a single dose of tocopherol and with 17% in the "unprotected" males. No differences in the mean values among the males were noted, regardless of whether 1, 5 or 27 mg alpha tocopherol had been given.

While it seems fairer to compare body weight deficits rather than body weight averages, it is to be emphasized that both computations lead to the same conclusions.

II. Effect on testes

In figure 4, the weights of one testis of rats on the "Evans-Burr" diet are presented in relation to age and body weight deficit. As previously described (Kaunitz et al., '44; Pappenheimer et al., '44), a single dose of tocopherol given on the fifteenth day delays the onset and decreases the degree of testicular atrophy.

[4] This colony had been raised from 1941 to 1944 in cooperation with Dr. A. M. Pappenheimer and Mrs. Claudia Schogoleff.

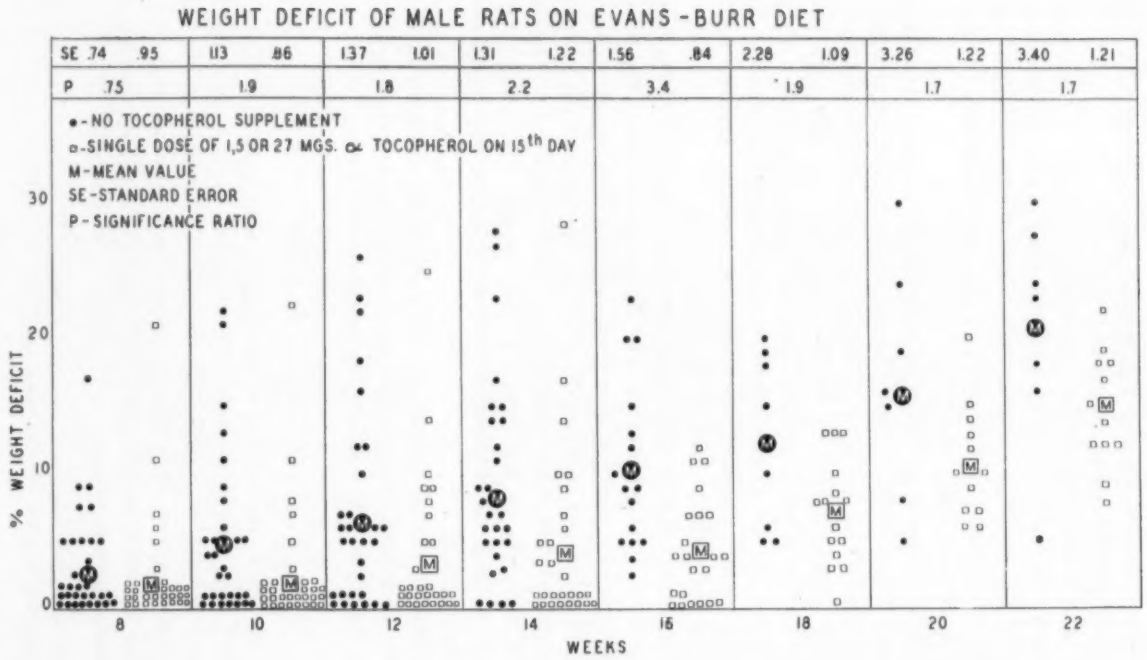
WEIGHT DEFICIT OF MALE RATS ON EVANS-BURR DIET
SE .74 .95 1.13 .86 1.37 1.01 1.31 1.22 1.56 .84 2.28 1.09 3.26 1.22 3.40 1.21
P .75 1.9 1.8 2.2 3.4 1.9 1.7 1.7
•-NO TOCOPHEROL SUPPLEMENT
▫-SINGLE DOSE OF 1,5 OR 27 MGS. α TOCOPHEROL ON 15th DAY
M-MEAN VALUE
SE-STANDARD ERROR
P-SIGNIFICANCE RATIO
% WEIGHT DEFICIT
0
10
20
30
8
10
12
14
16
18
20
22
WEEKS

Figure 3

In the age group of 60 to 70 days, only one animal out of five with a testicular weight of less than 700 mg had sustained a moderate body weight deficit. In the age group from 90 to 120 days, fourteen out of twenty-nine animals with testicular weights lower than 800 mg had grown optimally. Body weight

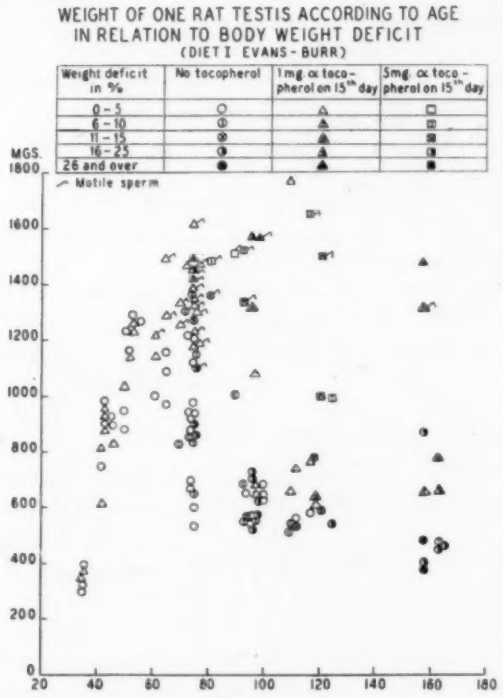

Figure 4

deficit, therefore, did not precede the testicular atrophy in at least 50% of the animals with atrophied testes.

Figure 5 demonstrates the weights of one testis of rats on the semi-synthetic diet in relation to age and body weight. The proportion of animals with atrophied testes and simultaneous body weight deficit is, in the age groups of 70–80 days,

greater than it was among the "Evans-Burr" rats, but there are also in this group animals that had pronounced testicular atrophy despite optimal growth; on the other hand, the number of rats with testes of normal weight and concurrent body weight deficit is considerable. Therefore, this series, too, indicates that body weight deficit does not necessarily precede the testicular atrophy.

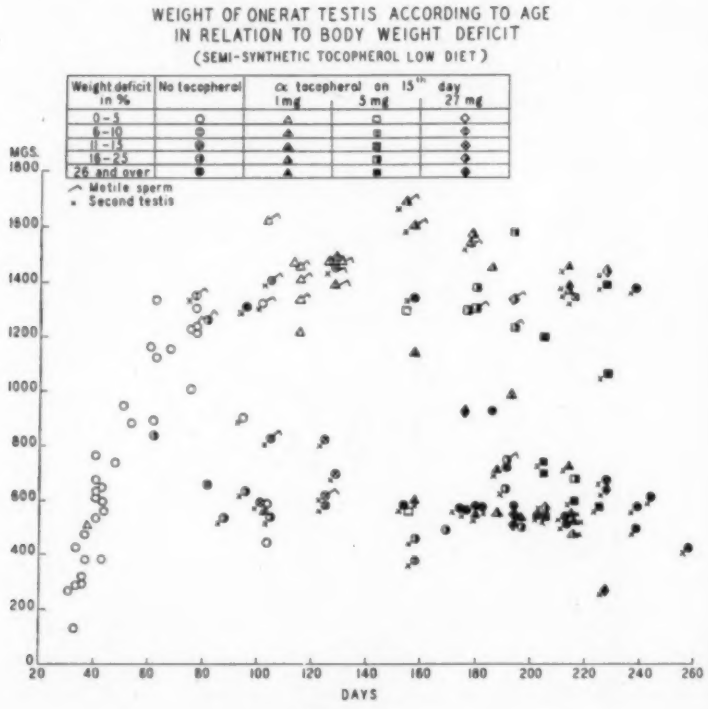

Figure 5

DISCUSSION

Storage and slow utilization of tocopherol cannot fully explain the protracted effect of a single dose of alpha tocopherol, because in the case of the testes (Kaunitz et al., '44;

Pappenheimer et al., '44), it could be proved that the same amount of tocopherol had different effects when given at different ages, and in this study it was observed that doses of from 1 to 27 mg had the same effect on body weight and delay of testicular atrophy when given at the fifteenth day of life. On the "Evans-Burr" diet, however, the degree of testicular degeneration was roughly proportional to the dose given on the fifteenth day. Thus far no explanation can be offered for this latter discrepancy.

If only storage were responsible for the effect of a single dose of tocopherol, one would expect a more pronounced influence from a higher dose and there could not be much difference between the effect of the administration on the sixth and fifteenth days.

The weight deficit caused by the absence of tocopherol does not necessarily antedate the occurrence of testicular atrophy. This permits the conclusion that weight deficit and testicular atrophy are two different, dissociated consequences of tocopherol deficiency in rats.

SUMMARY AND CONCLUSIONS

1. The body weight deficit below the curve of optimal growth was determined in albino rats kept on two different tocopherol low diets.

2. A single dose of alpha tocopherol of 1, 5 or 27 mg administered on the fifteenth day of life to the nursing young significantly reduces the degree of body weight deficit produced by vitamin E deficiency after the seventh week.

3. Determination of the testicular weight in relation to the body weight deficit indicated that testicular atrophy often occurs before body weight deficit is present. Weight deficit and testicular atrophy are therefore dissociated effects of vitamin E deficiency.

4. These observations suggest that in rats there is probably a critical need for tocopherol during the third week of life, since administration of tocopherol during that period exercises a prolonged effect.

ACKNOWLEDGMENT

We wish to acknowledge with gratitude Dr. Alwin M. Pappenheimer's helpful suggestions regarding this work.

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EVIDENCE OF CITRIC ACID SYNTHESIS IN HUMAN SUBJECTS

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ONE FIGURE

(Received for publication April 4, 1946)

It is known from work on experimental animals that citric acid is synthesized in the animal body (Sherman, Mendel and Smith, '36a; Smith and Meyer, '39; Schroeder and Smith, '43; and Class and Smith, '43). No observations of similar synthesis in humans have been reported. An experiment designed primarily to study thiamine (Hathaway and Strom, '46) and riboflavin (Hathaway and Lobb, '46) synthesis and excretion offered the opportunity to investigate citrate excretion on a diet containing no citrates, and on a controlled citrate intake. This paper reports observations which have bearing on the subject of citric acid synthesis in humans.

EXPERIMENTAL

Three women were maintained on a synthetic diet free of citrates for 45 days. After a month's respite they were given, for 21 days, a controlled diet which supplied small amounts of citrates from natural foods. The diets were of similar nutritive value and were considered to be adequate in all respects. Details concerning the subjects and diets have been reported by Hathaway and Strom ('46). Twenty-four-hour urine specimens were preserved with enough glacial acetic acid to maintain an acid concentration of 2 or 3%. Samples were stored under refrigeration until analyzed. The citrate

content of the samples was determined by the method of Pucher, Vickery and Leavenworth ('34). No fecal analyses for citrates were made since it has been shown by animal experiments that the feces contain very small and relatively constant amounts (Sherman et al., '36b; Kuether et al., '40).

RESULTS AND DISCUSSION

The values for daily excretions of citrates as citric acid are given in table 1. On the synthetic diet these values varied from 563 to 1179 mg for subject A; 673 to 994 mg for subject B; and 437 to 1019 mg for subject C. Citrate excretion on the

TABLE 1

Daily citric acid excretion by subjects A, B and C on synthetic and natural food diets.

SYNTHETIC DIET								NATURAL FOOD DIET			
Day	Mg citric acid per day			Day	Mg citric acid per day			Day	Mg citric acid per day		
	A	B	C		A	B	C		A	B	C
1	874	673	836	24	955	797	726	1	506	805	968
2	839	742	891	25	925	900	835	2	614	673	945
3	958	806	862	26	922	929	711	3	713	790	853
4	786	808	725	27	940	884	756	4	910	666	809
5	816	764	746	28	912	813	729	5	637	689	772
6	947	759	731	29	903	769	823	6	675	672	847
7	808	781	744	30	1175	844	1019	7	730	710	627
8	789	862	851	31	1179	914	933	8	680	637	729
9	900	778	892	32	1008	825	820	9	772	681	742
10	805	865	866	33	1001	884	864	10	892	745	695
11	667	861	771	34	1083	850	853	11	706	660	691
12	703	907	737	35	1048	981	798	12	704	643	651
13	563	910	766	36	1006	908	879	13	805	761	527
14	662	927	610	37	1012	923	786	14	760	818	481
15	733	918	437	38	797	850	759	15	771	822	552
16	686	819	526	39	850	975	781	16	871	769	494
17	643	862	589	40	869	954	784	17	928	924	633
18	723	859	672	41	813	960	672	18	733	825	488
19	704	893	582	42	787	804	623	19	832	983	632
20	803	943	622	43	920	994	848	20	888	1005	649
21	778	971	677	44	650	961	761	21	735	827	715
22	820	790	621	45	800	926	871				
23	918	773	731								

natural-food diet differed very little from that on the synthetic diet, the daily excretion varying from 506 to 928 mg for subject A; 637 to 1005 mg for subject B; and 481 to 968 mg for subject C. The natural-food diet was shown to contain an average of 797 mg citric acid per day. When this amount of extra citric acid was ingested, urinary excretion of citrates was not affected; nor was it affected when the dietary constituents were supplied as natural foods rather than in the highly purified forms of the synthetic diet. Individual variations in daily citrate excretion of 300 to 500 mg were observed on the rigidly controlled synthetic diet as well as on the natural-food diet. This indicates that in this study citrate excretion was influenced by other factors, independent of dietary constituents.

Relation of citrate excretion to the menstrual cycle

In figure 1 the daily citrate excretion for each subject when on the synthetic diet is plotted graphically to show its relationship to the menstrual cycle. The excretions for subjects A and C show definite cyclic variations, but those of subject B show less marked cyclic variation. In each case the highest excretions occurred about midway in the menstrual cycle and the lowest excretions during menstruation. The midmenstrual peaks exceeded the menstrual levels of excretion by 616 mg, 321 mg, and 583 mg for subjects A, B, and C, respectively. These results confirm those of Shorr, Bernheim and Taussky ('42) who first observed the relationship between citrate excretion and the steroidal reproductive hormones. This hormonal factor may well account for the large individual variations observed in the present study.

Citrate excretion after test doses of ascorbic acid

Purinton and Schuck ('43) observed an inverse relationship between the retention of ascorbic acid by the body and the excretion of citric acid. In connection with another part of the present study (Delaney, '45) test doses of 400 mg of

ascorbic acid were given orally on the forty-second, forty-third, and forty-fourth days of the synthetic diet. The ascorbic acid retention decreased with successive doses, but no variations in citrate excretion were observed beyond those which can be explained by relationship to the menstrual cycle.

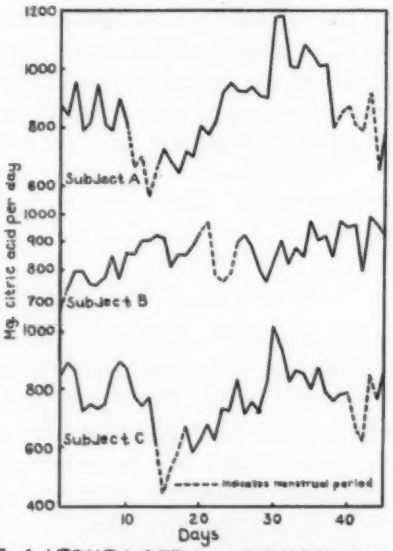

Fig 1. URINARY CITRIC ACID EXCRETION AS RELATED TO THE MENSTRUAL CYCLE

Evidence of citric acid synthesis

The total amounts of citrates as citric acid excreted during the 45-day period on the synthetic diet were 38, 39 and 34 gm for subjects A, B, and C, respectively. This citric acid was necessarily of endogenous origin since there was no dietary source of citrates. Sherman, Mendel and Smith ('36a), Smith and Meyer ('39) and Schroeder and Smith ('43) have reported similar results for balance studies with animals. Endogenous citric acid was considered to be synthesized by the body until Dickens ('41) found that ox and kitten bone con-

tained nearly 500 mg citric acid per 100 gm of fresh bone, and indicated that bone stores had not been ruled out as its source. Class and Smith ('43) and Leonards and Free ('44) demonstrated in animal experiments that the urinary citrates did not originate from reserve stores. A rough calculation can be made of possible bone stores of citric acid in the human. Assuming that the fresh weight of the skeleton of the human adult is 15% of the body weight (Mitchell et al., '45) and that human bone contains citric acid in amounts comparable to that reported by Dickens ('41) for animal bone (approximately 0.5%), subjects A, B, and C would have stores amounting to approximately 43, 45 and 47 gm, respectively. If these stores were the source of the urinary citrates they would have been from 70 to 90% depleted by the end of the dietary period. No decline was found in the level of excretion toward the end of the period which could be attributed to decreasing body stores. Such a great depletion of the citric acid stores in the bones would probably have caused some increase in calcium excretion since the two substances are thought to be closely associated in bone (Dickens, '41; Gomori and Gulyas, '44). No such change in calcium balance was noted.[1] Therefore the results of this study with human subjects support the conclusion drawn from animal experiments that citrates in the urine are largely synthesized by tissues rather than coming from body stores.

SUMMARY AND CONCLUSIONS

Three normal women were maintained for 45 days on a synthetic diet free from citrates and for 21 days on a controlled natural-food diet of similar nutritive value but supplying approximately 797 mg citric acid per day. Urinary excretions of citrates were followed daily.

Individual variations in daily citrate excretion of 300 to 500 mg were found on both the synthetic and the natural-food diets.

[1] A report of the calcium, phosphorus and nitrogen metabolism of the subjects on the synthetic and natural-food diets is in preparation.

Variations in citrate excretion were found to bear a definite relationship to the phases of the menstrual cycle.

After oral administration of test doses of 400 mg of ascorbic acid, no variations in citrate excretion which could be correlated with decreasing ascorbic acid retention were observed.

Subjects A, B, and C excreted a total of 38, 39, and 34 gm, respectively, of citric acid while on the synthetic diet. Synthesis by the tissues is believed to be the explanation for these relatively large amounts of endogenous citric acid.

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